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PRINCIPAL INVESTIGATOR: Xiaoliu Zhang, M.D., Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, TX 77030

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Xiaoliu Zhang, M.D., Ph.D.

E-Mail: xzhang@bcm.tmc.edu

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14. ABSTRACT

The underlying hypothesis for this project is that incorporation of cell membrane fusion function into an oncolytic herpes simplex virus (HSV) can significantly enhance the anti-tumor effect of the virus. Three specific aims have been proposed and they are: 1) to demonstrate that fusogenic oncolytic HSVs are a potent anti-tumor agent for advanced ovarian cancer; 2) to prove that fusogenic oncolytic HSVs have the same safety profile as their non-fusogenic counterparts; 3) to explore novel delivery strategies that can evade host's anti-viral immunity for repeated delivery. During the funding period, these aims have been mostly achieved. Our data show that incorporation of a fusogenic glycoprotein (GALV.fus) into an oncolytic herpes simplex virus through a novel controlling mechanism can significantly enhance the antitumor potency of the virus without significantly increasing its toxicity. In the efforts to identify a strategy that can deliver an oncolytic HSV to tumor tissues systemically in the presence of host's antiviral immunity, we demonstrate that delivery of an oncolytic HSV as a DNA form rather the traditional viral particles represents a possibility. We also show that blood cells such as monocytes/macrophages from STAT1-deficient background could function as cell carriers for systemic delivery of these oncolytic viruses. Several publications have been produced during the funding period. One of the oncolytic viruses that were studied during the funding period is currently being evaluated for translation into clinical testing for treating solid tumors including ovarian cancer.

15. SUBJECT TERMS
Oncolytic virus, advanced ovarian cancer, experimental therapy, syncytial formation

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INTRODUCTION

Because of its inconspicuous early symptoms and the lack of effective screening techniques, ovarian cancer frequently presents at an advanced stage (III/IV), in which the disease has usually spread to the peritoneal cavity. Current therapy for advanced-stage ovarian cancer consists of debulking surgery followed by chemotherapy. Although the clinical response rate is 60–70%, most patients ultimately relapse and succumb to recurrent chemoresistant disease, leading to 14,000 deaths in the United States each year. Hence, there is an urgent need for novel therapeutics that can provide significant clinical benefits or cure for patients with advanced-stage ovarian cancer. Replication-competent herpes simplex virus (oncolytic HSV) holds considerable promise for treating malignant solid tumors such as ovarian cancer, although the potency of the virus needs improvement if its full clinical potential is to be realized. We recently demonstrated that addition of a cell membrane fusion capability to an oncolytic HSV can significantly increase the antitumor potency of the virus [1, 2]. The modified virus kills tumor cells efficiently and directly through both replication and cell membrane fusion. In this funded project, we propose to evaluate the antitumor potency of a newer version of the fusogenic oncolytic HSV, in which two membrane fusion mechanisms were incorporated into a single virus (Synco-2D), against ovarian cancer. Specifically, Three specific aims have been proposed and they are: 1) to demonstrate that fusogenic oncolytic HSVs are a potent anti-tumor agent for advanced ovarian cancer; 2) to prove that fusogenic oncolytic HSVs have the same safety profile as their non-fusogenic counterparts; 3) to explore novel delivery strategies that can evade host's anti-viral immunity for repeated delivery. As detailed in the following sections, we believe that these aims have been mostly achieved during the funding period.

BODY

1. In vitro antitumor activity of doubly fusogenic oncolytic HSV against human ovarian cancer cells

Viral stocks were prepared by infecting Vero cells with 0.01 plaque-forming units (pfu) per cell. When

cells showed complete cytopathic effect, heparin (Sigma, St. Louis, MO) was added to the culture medium at a final concentration of 50 μg/ml) and cells were cultured for another 3 h to release the virus into the medium. The medium was then collected and was subjected to a low speed centrifugation at 1,000 *g* for 10 min. The cleared supernatant was transferred to another tube and the virus was pelleted through high speed centrifugation (29,000 *g* for 4 h). The viral pellet was resuspended in PBS containing 10% glycerol and stored at -80^{0}C.

We phenotypically characterized the viruses in greater detail on two human ovarian cancer cell lines (Hey-8 and SKOv3), infected with either Baco-1 or Synco-2D at 0.001 pfu/cell. As shown in Fig. 1, the syncytial plaques after Synco-2D infection differed strikingly from the usual round-cell plaques derived from infection with Baco-1. By 40 h, the Synco-2D-infected tumor cells had fused together, forming a dense material (middle of the infectious focus) that was separated from the boundary of the plaque by a large gap area. This distinctive pattern of syncytial development was especially prominent in Hey-8 cells.

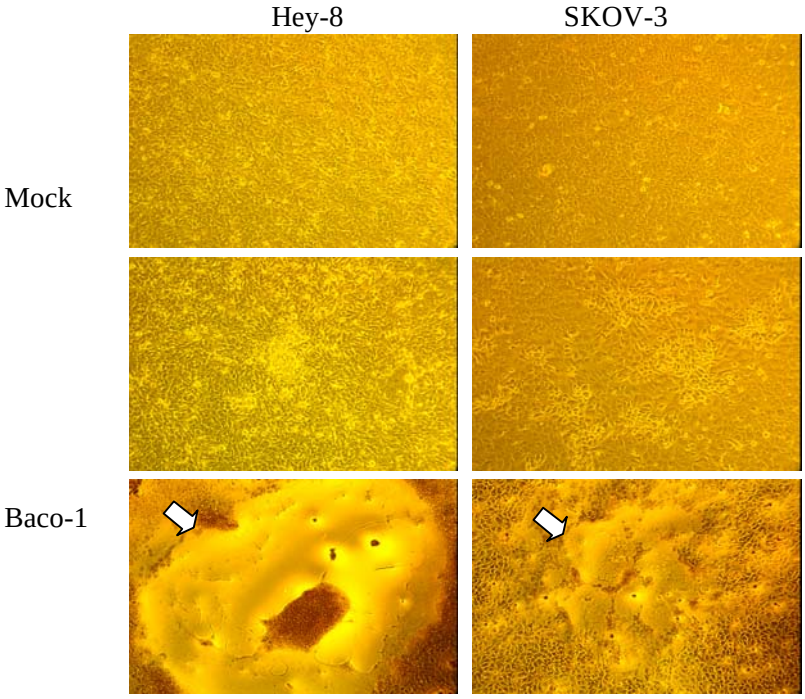

Fig. 1. *In vitro* phenotypic characterization of Synco-2D in ovarian cancer cells. Hey-8 or SKOv3 ovarian cancer cells were infected with either Baco-1 or Synco-2D at 0.01 pfu/cell. Photomicrographs were taken at 48 h after viral infection (original magnification, x200). Arrows indicate a single syncytium.

To determine if the marked syncytial formation resulting from Synco-2D infection would enhance tumor cell killing, we infected ovarian cancer cells with Baco-1 or Synco-2D at a relatively low multiplicity of infection (0.1 or 0.01 pfu/cell), permitting us to assess both the inherent cytotoxicity of the input virus as well as the ability of the virus to replicate and spread in these cells. The cytotoxic effect of the viruses was quantified by calculating the percentage of viable cells remaining in the wells after infection. The results (Fig. 2) showed a significantly greater tumor cell killing effect by Synco-2D compared to Baco-1 (p<0.01, all comparisons). At 0.01 pfu/cell, Synco-2D reduced the viable cells to less than 40% within 24 h (Fig. 2A). At 0.1 pfu/cell, it completely destroyed the Hey-8 tumor cells within 48 h, a time where there was still more than 30% viable tumor cells in the well infected with Baco-1 (Fig. 2B).

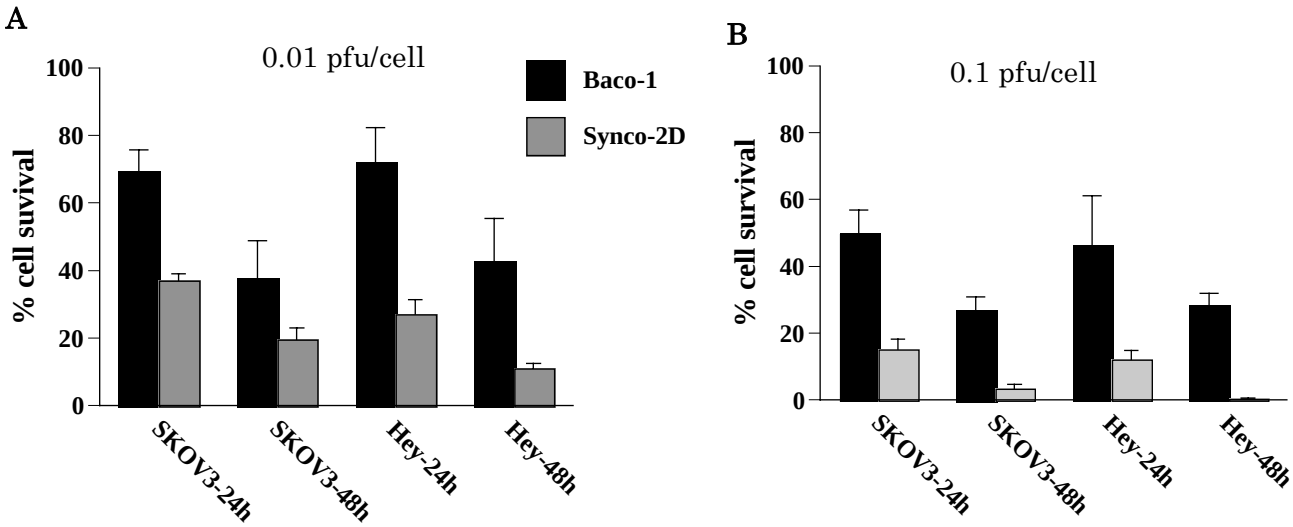

Fig. 2. Comparison of the cytotoxicity of Baco-1 and Synco-2D in cultured ovarian cancer cells. Hey-8 or SKOv3 ovarian cancer cells were seeded into 24-well plates and infected with Baco-1 or Synco-2D at 0.01 (A) or 0.1 (B) pfu/cell. Cells were collected at 24 h or 48 h after infection, stained with trypan blue and counted. The percentage of cell viability was determined by dividing the number of viable cells from the infected well by the number of cells from an uninfected well. The data are reported as means ± standard deviations. All comparisons shoed a significant advantage in cytotoxicity for Synco-2D ($p < 0.01$).

3. Therapeutic effects of Synco-2D on peritoneal metastases of ovarian cancer

Peritoneal invasion of ovarian cancer is a common and serious clinical problem. It has been reported that about 70% of late-stage ovarian cancer patients have metastatic disease in the peritoneal cavity. We therefore chose a peritoneal metastasis model (xenografted Hey-8 cells) as means to testing the efficacy of Synco-2D against human ovarian cancer. Freshly harvested Hey-8 cells were inoculated into the peritoneal cavities of nude mice at a dose of 3×10^5 per mouse. Two weeks later, palpable tumor had formed near the injection site in all mice. The average tumor diameter was approximately 3 mm. At this time, mice were injected intraperitoneally with 2×10^7 pfu/200 µl of either Baco-1 or Synco-2D, or PBS (control), at a site distant from that of tumor cell implantation. A second intraperitoneal injection with the same amount of virus was given 2 weeks later. During the interim period, PBS control mice began to die from tumor or had to be euthanized due to tumor progression and cachexia. Thus, the mean survival time in this group was 36.5±0.7 days (none of the mice survived), there was clear intraperitoneal dissemination of tumor, with an average of 2.5±0.9 tumor nodules found in regions distal from the site of the tumor implantation (Table 1). In the Baco-1 treatment group, 3 mice died before the end of the experiment (first death, 34 days after tumor cell implantation); 5 survivors bore a single large tumor when examined at necropsy. By contrast, none of the Synco-2D-treated mice died or were euthanized during the experiment. Strikingly, 6 of the 8 mice were entirely tumor-free at necropsy by the end of the experiment (Table 1). The other two animals had tumors that are significantly smaller than those in Baco-1-treated mice ($p<0.01$, Table 1).

Table 1. Results of Synco-2D therapy for xenografted human ovarian cancer in the peritoneum

Treatment	*N*	No. mice with tumors	No. of nodules	Tumor weight (g)[b]	Death rate
PBS	8	8/8	2.5±0.9	1.0±0.6	8/8
Baco-1	8	8/8	1.0±0.0[c]	1.5±0.7	3/8[c]
Synco-2D	8	2/8[a]	0.25±0.4[a]	0.1±0.3[a]	0/8[a]

[a] $p<0.01$ as compared with either PBS or Baco-1 treated group.

[b] Means and standard deviations.

[c] $p<0.01$ as compared with PBS control.

4. In vivo toxicity evaluation

4.1. Intracranial injection. As HSV is a neurotropic pathogen, it is therefore important to evaluate its potential toxicity in the CNS. Although the potential toxic effect of Synco-2D on the brain was evaluated after systemic delivery (see the next section), the presence of the blood-brain barrier may prevent the virus from efficiently getting into the brain from the blood stream. It is possible that the full extent of its neurotoxicity may not be comprehensively evaluated after systemic delivery of the virus. We therefore injected the viruses directly into mouse brain at a relatively high dose to examine its neurotoxicity in the following experiments.

Stereotactic injection of the virus solution (in the volume of 3 μl) into the brains of immune competent mice was done essentially according to the procedure as described [3]. Briefly, 8-week-old mice (BALB/c) were anesthetized and placed in a stereotactic frame (Stoelting). A hole was drilled in the skull 1 mm anterior and 2 mm lateral to the bregma with a 0.9-mm burr to expose the dura. The mice were then injected with 3μl of Hanks' buffered saline containing the designated amount of virus, using a 100μl syringe (Hamilton) fitted with a 26-gauge needle and connected to the manipulating arm of the stereotactic frame. The injection, given over 1 min, was directed to the caudate nucleus at a depth of 3.5 mm from the dura. The needle was left in place for 3 min and then withdrawn slowly over another minute, to prevent reflux of the virus solution. There were 3 groups (n=8) of mice in this experiment: Synco-2D, Baco-1 and PBS. Three mice from each group were sacrificed 2 days after virus injection and the fresh brain tissue was homogenized (in 0.5 ml medium) for virus titration. The remaining five mice from each group were kept for 3 weeks before they were sacrificed. The brain was removed,

fixed in formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin.

The virus titration result of the brain tissues was shown in Table 2. A typical brain section from each group of mice was shown in Fig. 3. The amount of infectious viruses retrieved from the brain tissues of mice receiving either Synco-2D or Baco-1 was significantly lower than the amount of viruses that was initially injected, indicating that the oncolytic HSVs did not replicate significantly in the brain tissue. In addition, there was no significant difference on the amounts of the virus recovered from brain tissues of Synco-2D and Baco-1-treated groups. During the observation period, no animal from either group died. The brain sections show no obvious abnormality at the time (3 weeks) after virus inoculation. Together, these results indicate that Synco-2D was not significantly more toxic than the nonfusogenic Baco-1 when it was inoculated directly into the CNS.

Table 2. Quantification of oncolytic HSVs after intracranial administration

	PBS	Baco-1	Synco-2D
Virus titer (pfu/ml)	<10pfu*	$1.5\pm0.2\text{x}10^2$	$2.1\pm0.3\text{x}10^2$

* As the lowest virus dilution of the homogenized tissue samples was 1:5 (i.e., adding 100 µl of the homogenized tissue supernatant to 400 µl of DMEM to make a total of 500 µl for infection of Vero cell monolayer seeded in 6-well plates). So if no virus plaque was detected from the well, then the amount of the infectious virus in the organ was <10 pfu/ml.

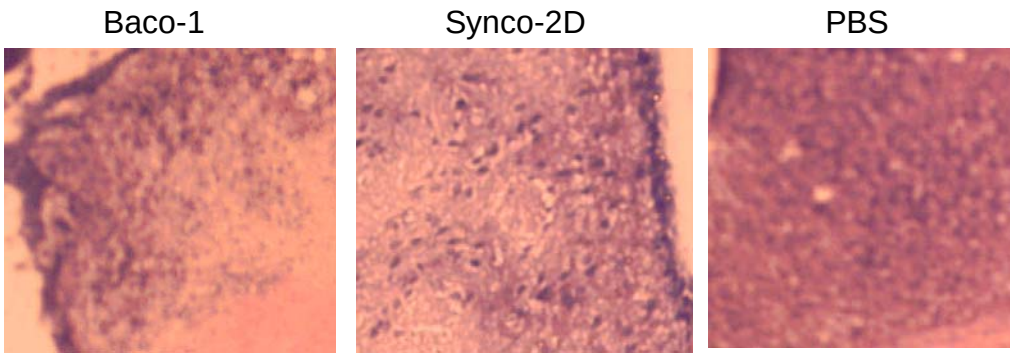

Fig. 3. Histology of brain tissues after intracranial delivery of oncolytic HSVs. The brain sections were prepared from the indicated mice at 3 weeks after virus delivery. Original magnification: 200X.

4.2. Systemic injection. Even when intratumorally delivered, it is still possible that the oncolytic HSVs may leak from the tumor site into the blood stream. It is therefore important to assess the toxicity of Synco-2D after systemic delivery. Six-week-old immune competent BALB/c mice were injected through the tail vein with either the doubly fusogenic Synco-2D or Baco-1 at two different doses: $1X10^7$ or $1X10^8$ pfu, or merely PBS as a negative control. There were 13 mice in each group, 5 for evaluating virus distribution/histological examination and the other 8 for determining mortality.

Toxicity evaluation criteria:

1) Incidence of mortality. For evaluation of mortality after oncolytic HSV administration, animals were observed for a six-month period. During the observation period, one mouse from the groups receiving the higher dose of Synco-2D and Baco-1 died during the acute phase of virus infection (at day 2 and day 3 after virus infusion, respectively). No serious disease symptom was noticed afterwards during the observation period (Table 3).

Table 3. Mortality after systemic administration of oncolytic HSVs

Viruses	Dose	Route admin.	No. mice	mortality
Baco-1	$1X10^7$	i.v.	6	0
Baco-1	$1X10^8$	i.v.	6	1
Synco-2D	$1X10^7$	i.v.	6	0
Synco-2D	$1X10^8$	i.v.	6	1
PBS	200 μl	i.v.	6	0

2) Histopathological findings after systemic administration of oncolytic HSVs and quantification of retrievable virus from the major organs

For the purpose of determining histological abnormality, mice were euthanized 5 days after virus administration. Major organs were collected and were cut into half. One half was immediately homogenized and the lysate was used for titration on Vero cell monolayers to determine the virus distribution. Another half of the organ tissues was embedded in paraffin and sections were prepared for histological examinations. Only infrequent mild histological abnormality was noticed in liver sections from mice receiving administration of either Baco-1 or Synco-2D (scored as either +). No obvious pathological changes were identified in any other organs (Table 4). The virus titration results from the homogenized organ tissues (Table 5) showed that only a small quantity of infections virus could be detected from liver, which is the major site of virus deposit after systemic delivery. No infectious virus could be detected from any other major organs. These results demonstrate that systemic administration of either the doubly fusogenic Sync-2D or the nonfusogenic Baco-1 only causes a mild and transient liver damage and Synco-2D does not seem to be significantly more toxic than Baco-1 to the liver. This mild liver damage is probably due to the preferred deposit of viruses to the liver after systemic delivery.

Table 4. Results of histological examination after systemic administration of oncolytic HSVs

Organs	**PBS**	**Baco-1**		**Synco-2D**	
	$100\mu l$	$1X10^7$	$1X10^8$	$1X10^7$	$1X10^8$
Liver	—	+	+	—	+
Kidney	—	—	—	—	—
Pancreas	—	—	—	—	—
Heart	—	—	—	—	—
Lung	—	—	—	—	—
Spleen	—	—	—	—	—
Brain	—	—	—	—	—

Table 5. Infectious virus distribution in major organs after systemic administration of oncolytic HSVs

Organs	PBS	Baco-1		Synco-2D	
	100μl	1X10^7	1X10^8	1X10^7	1X10^8
Liver	<10pfu/ml$^{\Psi}$	<10pfu/ml	22.6±4.5pfu/ml	<10pfu/ml	18.3±5.7pfu/ml
Kidney	<10pfu/ml	<10pfu/ml	<10pfu/ml	<10pfu/ml	<10pfu/ml
Pancreas	ND*	<10pfu/ml	<10pfu/ml	<10pfu/ml	<10pfu/ml
Heart	ND	<10pfu/ml	<10pfu/ml	<10pfu/ml	<10pfu/ml
Lung	<10pfu/ml	<10pfu/ml	<10pfu/ml	<10pfu/ml	ND
Brain	<10pfu/ml	<10pfu/ml	<10pfu/ml	<10pfu/ml	<10pfu/ml
Spleen	<10pfu/ml	<10pfu/ml	<10pfu/ml	<10pfu/ml	<10pfu/ml

$^{\Psi}$As the lowest virus dilution of the homogenized tissue samples was 1:5 (i.e., adding 100 µl of the homogenized tissue supernatant to 400 µl of DMEM to make a total of 500 µl for infection of Vero cell monolayer seeded in 6-well plates). So if no virus plaque was detected from the well, then the amount of the infectious virus in the organ was <10 pfu/ml;

*Not done, due to bacteria contamination of the samples.

5. Studies of antiviral immune responses and development of strategies to evade them

5.1. Anti-HSV immune serum can't prevent spread of fusogenic oncolytic HSV in vitro

To compare the effects of anti-HSV immune serum on the spread of fusogenic and non-fusogenic oncolytic HSV in vitro, we initially infected tumor cells (in 12-well plates) with either Synco-2D or Baco-1 at 0.01 pfu/cell for 1 h. Then, medium containing pooled mouse anti-HSV sera at 1: 50 dilution, which completely blocked Baco-1 infectivity on Vero cells, was added to the wells. As shown in Fig. 4, inclusion of the immune sera had minimal effect on syncytial formation by Synco-2D, but greatly inhibited the spread of non-fusogenic oncolytic HSV. These findings support the underlying

hypothesis that the antitumor activity of fusogenic oncolytic HSV is less affected by antiviral immunity when compared to a conventional oncolytic HSV once the virus has entered into target cells.

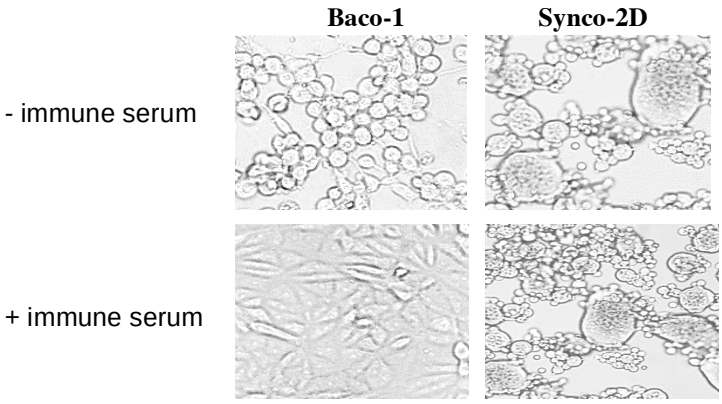

Fig. 4. Effect of anti-HSV immune sera on virus propagation and production. The photos on the left show the phenotypes and the table above gives the virus yields in tumor cells, with or without anti-HSV sera in the medium.

5.2. Liposome formulation of oncolytic HSVs.

Next we tested if formulating oncolytic HSVs with liposomes could evade the host's humoral antiviral immunity. We initially tested the condition of liposome formulation of oncolytic HSVs. We prepared the virus in 3 different forms: 1) DNA form of viral genome; 2) intact viral particles; 3) de-enveloped viral capsid. We then formulated these different forms of virus preparations with liposomes and examined their infectivity in vitro by counting the plaques after adding them onto Vero cell monolayers. The results showed that viral DNA formulated with liposomes gave the highest number of plaques. Viral capsids formulated with liposome gave the second best result. The intact viral particles formulated with liposome produced the lowest number of viral plaques, possibly because the intact viral particles already contain an outside lipid membrane, which may have prevented liposome formulation.

5.3. Delivery of oncolytic HSVs by carrier cells. We also tested if T-lymphocytes could act as a carrier for oncolytic HSV delivery. As T lymphocytes can freely circulate through the vascular system and can infiltrate to the metastatic tumors, once loaded with the oncolytic HSVs, they may be able to send the virus selectively to the tumor sites. Furthermore, the cell membrane may function as a protective shield to protect the virus from neutralizing antibodies. Human or murine T cells were infected with

Baco-1, which carries the green fluorescent protein (GFP) marker gene in its viral genome and its infectivity can thus be easily identified. The results showed either human or murine T lymphocytes were resistant to infection by oncolytic HSVs. Even at a multiplicity (MOI) of 10, less than 5% of cells showed GFP expression. We then examined NK cells and macrophages, another two cell types that are major components of circulating blood. In addition, both NK cells and macrophages have the ability to infiltrate to tumor tissues. We initially performed a similar in vitro experiment as described above to test the infectivity of the oncolytic Baco-1 on these cells. The results showed that both NK cells and macrophages from human and murine origins were also resistant to infection by Baco-1. At an MOI of 5, less than 10% of cells showed GFP expression.

Recently it has been reported that retrovirus can adhere nonspecifically, or 'hitchhike', to the surface of cytotoxic t lymphocytes (CTLs). CTLs hitchhiked with a retroviral vector can then move to tumor sites and “hand off” the viruses to tumor cells to initiate virus infection [4]. We thus tested this strategy to determine if oncolytic HSV could hitchhike to carrier cells that were found to be non-permissive to infection of HSV vectors in our previous experiments. We incubated either NK cells or macrophages of both human and murine origins ($1X10^5$) with $5X10^5$ plaque-forming-units (pfu) of oncolytic HSV Baco-1 for 1 h. The cells were then gently washed 2 times with PBS and were added to Vero cell monolayers. Viral plaques were examined 48 h later. The results showed that Baco-1 was efficiently loaded to the carrier cells in this way and was then successfully “handed off” to the testing cell monolayers. Since more than $1X10^5$ plaques were obtained from each preparation (with $1X10^5$ carrier cells), it was concluded that approximately 100% of the carrier cells were able to hand off the oncolytic virus. These results indicate that this hitchhike strategy may be a useful way for carrier cell-medicated delivery of oncolytic HSVs for the treatment of metastatic prostate cancer.

KEY RESEARCH ACCOMPLISHMENTS

- In vitro work showed that Synco-2D infection induces a distinctive syncytial phenotype and

a significantly enhanced killing activity against human ovarian cancer cells.

- Intraperitoneal injection of Synco-2D had a significantly better therapeutic effect against metastatic human ovarian cancer xenografts than the nonfusogenic HSV, leading to eradication of all tumor masses in 75% of the animals, whereas no animals in the conventional oncolytic HSV treated group was tumor-free.
- Direct injection of the virus to the CNS at a relatively large dose did not cause any mortality. Virus titration from the brain tissue 2 days after virus administration indicates that the viruses had very little replication capability in the brain, which is very important for its potential clinical application, as HSV is considered as neurotropic.
- Systemic administration of oncolytic HSVs caused a mild liver abnormity as identified by histological examination. However, there was no significant difference on the severity of liver toxicity between the doubly fusogenic and the nonfusogenic oncolytic HSVs. This mild liver toxicity is most likely due to the preferred deposit of the systemically delivered viruses to this organ. Virus titration did not detect any residual infectious virus from other major organs.
- Fusogenic oncolytic HSVs have the ability to spread to surrounding tumor cells even in the presence of high concentration of antiviral immune sera, indicating the ability of this unique type of oncolytic HSV to evade the host's acquired antiviral immunity once the virus has entered into the target cells.
- Oncolytic HSVs could be formulated with liposomes for in vivo delivery. Especially, preparation of oncolytic HSVs in the DNA or de-enveloped capsid form could be more efficiently formulated with liposomes than the intact viral particles. Thus, these two forms of viral preparation will be used in the future in vivo studies.
- Our data demonstrate that macrophages could function as carrier cells for delivery of oncolytic HSVs by the recently reported "Hitchhike" strategy. This will likely represent a

very efficient way of loading oncolytic virus to the carrier cells, which are otherwise non-permissive to infection of oncolytic HSVs.

REPORTABLE OUTCOMES

1. Conference presentation: The 6th Annual Meeting of the American Society of Gene Therapy (6/5-9/2003, Washington, DC). Title of abstract: Effective Therapy of metastatic ovarian cancer with an oncolytic herpes simplex virus incorporating two membrane-fusion mechanisms.

2. Conference presentation: The 9th Annual meeting of Japanese Society of Gene Therapy (7/19-20/2003, Tokyo, Japan). Title of abstract: Enhancement of the Therapeutic Efficacy of an Oncolytic Herpes Simplex Virus (HSV) by Two Membrane-Fusion Mechanisms: Comparison with a Conventional HSV Therapy.

3. Publication. Nakamori, M. Fu, X. Meng, F. Jin, A. Bast, R and **X**. **Zhang** (2003). Enhanced therapy of metastatic ovarian cancer with a double fusion oncolytic herpes simplex virus. *Clin. Cancer Res*. 9(7): 2727-2733.

4. Conference presentation: Dr. Zhang was one of three invited overview speakers at the 29th International Herpesvirus Workshop (held at Reno, Nevada, July 25-30, 2004). Title of talk: HSV vectors for gene therapy of solid tumors and genetic diseases.

5. Dr. Zhang and his associates were invited to contribute a book chapter on their work on developing fusogenic oncolytic HSVs for treatment of solid tumors including prostate and ovarian cancers. X. Fu, M. Nakamori and X. Zhang. (2005). Fusogenic Oncolytic Herpes Simplex Virus. In: Virus Therapy of Human Cancers. Sinkovics & Horvath eds. (New York: marcel dekker Inc.). pp713-738.

6. Publication. Xinping Fu, Lihua Tao and **Xiaoliu Zhang** (2007). A novel oncolytic virus derived from type 2 herpes simplex virus has potent therapeutic effect against metastatic ovarian cancer. *Cancer Gene Therapy.* 14: 480-487.

7. Dr. Zhang was invited to give a platform presentation at the 4th International Conference on Oncolytic Viruses as Cancer Therapeutics, held at Carefree, Arizona, March 14-17, 2007. He reported the most recent findings from his lab, including studies from this project.

CONCLUSIONS

1. The doubly fusogenic Synco-2D is an effective therapeutic agent against advanced ovarian cancer.

2. Despite its dramatically increased antitumor effect, Synco-2D is as safe as the first generation oncolytic HSV when extensively evaluated in vivo.

3. Novel delivery strategies, such as liposome formulation of viral DNA or capsid instead of intact viral particles, may evade host's antiviral immunity. Additionally, oncolytic HSVs may be systemically delivered by carrier cells such as lymphocytes and macrophages, in the presence of host's anti-HSV immunity.

REFERENCES

1. Fu, X. & Zhang, X. Potent systemic antitumor activity from an oncolytic herpes simplex virus of syncytial phenotype. Cancer Res. 62, 2306 - 2312 (2002).
2. Fu, X. et al. Expression of a fusogenic membrane glycoprotein by an oncolytic herpes simplex virus provides potent synergistic anti-tumor effect. Mol. Ther. 7, 748-754 (2003).
3. Chen, S. H., Shine, H. D., Goodman, J. C., Grossman, R. G. & Woo, S. L. Gene therapy for brain tumors: regression of experimental gliomas by adenovirus-mediated gene transfer in vivo. Proc Natl Acad Sci U S A 91, 3054-7. (1994).
4. Cole, C. et al. Tumor-targeted, systemic delivery of therapeutic viral vectors using hitchhiking on antigen-specific T cells. Nat Med 11, 1073-81 (2005).

Effective Therapy of Metastatic Ovarian Cancer with an Oncolytic Herpes Simplex Virus Incorporating Two Membrane Fusion Mechanisms[1]

Mikihito Nakamori, Xinping Fu,[2] Feng Meng, Aiwu Jin, Lihua Tao,[2] Robert C. Bast, Jr., and Xiaoliu Zhang[3]

Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, Texas 77030 [M. N., X. F., F. M., A. J., L. T., X. Z.], and The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030 [R. C. B.]

ABSTRACT

Purpose and Experimental Design: **Replication-competent herpes simplex virus [HSV (oncolytic HSV)] holds considerable promise for treating malignant solid tumors, although the potency of the virus needs improvement if its full clinical potential is to be realized. Incorporation of membrane fusion capability into an oncolytic HSV, either by screening for a syncytial HSV mutant after random mutagenesis or by inserting a hyperfusogenic glycoprotein from gibbon ape leukemia virus into the viral genome, can significantly enhance the antitumor effects of the virus (X. Fu and X. Zhang, Cancer Res., *62:* 2306–2312, 2002; X. Fu *et al.*, Mol. Ther., in press, 2003). We reasoned that both fusogenic strategies, incorporated into a single oncolytic HSV, might significantly improve virotherapy for ovarian cancer.**

Results: In vitro **characterization of a doubly fusogenic oncolytic HSV (Synco-2D) showed that this virus produces a distinctive syncytial phenotype, leading to a significantly increased tumor cell killing ability, compared with that of a nonfusogenic virus. When injected directly into the abdominal cavity of mice bearing human ovarian cancer xenografts, Synco-2D eradicated all tumor masses in 75% of the animals, whereas no animals in the conventional oncolytic HSV-treated group were tumor free.**

Conclusions: **This newly generated fusogenic oncolytic HSV is a promising candidate for clinical testing against advanced ovarian cancer.**

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[1] Supported by institutional start-up funds.
[2] Present address: The Fifth Affiliated Hospital of Zhongshan University, Zhuhai, China.
[3] To whom requests for reprints should be addressed, at Center for Cell and Gene Therapy, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Phone: (713) 798-1256; Fax: (713) 798-1230; E-mail: xzhang@bcm.tmc.edu.

INTRODUCTION

EOC[4] is the leading cause of death from gynecological malignancies. An estimated 23,400 new cases of EOC are diagnosed in the United States each year (1). Because of its inconspicuous early symptoms and the lack of effective screening techniques, this disease frequently presents at an advanced stage (III/IV), in which the disease has usually spread to the peritoneal cavity (2). Current therapy for advanced-stage ovarian cancer consists of debulking surgery followed by chemotherapy (3). Although the clinical response rate is 60–70%, most patients ultimately relapse and succumb to recurrent chemoresistant disease, leading to 14,000 deaths in the United States each year (1). Hence, there is an urgent need for novel therapeutics that can provide significant clinical benefits or cure for patients with advanced-stage EOC.

One experimental therapy that holds great promise for solid tumors such as ovarian cancer is the use of replication-competent (oncolytic) HSV (4, 5). These viruses can infect, replicate in, and kill tumor cells by a direct cytopathic effect, while showing only restricted ability to replicate in normal cells (6–8). In early clinical trials, however, treatment with the current generation of oncolytic viruses did not significantly affect tumor growth or improve prognosis (9, 10). This suboptimal result may reflect viral gene deletions, which can reduce the replicative potential of viruses in tumor cells. For example, deletion of the γ*34.5* gene significantly reduced viral growth even in rapidly dividing cells (8, 11–13). Hence, improvements in the potency of these oncolytic viruses are required to obtain clear benefits in cancer patients.

We recently reported that addition of a cell membrane fusion capability to an oncolytic HSV can significantly increase the antitumor potency of the virus (14, 15). The modified virus kills tumor cells efficiently and directly through both replication and cell membrane fusion. These two cytolytic mechanisms may also produce a synergistic effect through syncytial formation that facilitates the spread of the oncolytic virus in tumor tissue. The fusogenic activity of the virus was generated by random mutagenesis of a well-characterized oncolytic HSV (14) or by inserting a hyperfusogenic membrane glycoprotein from gibbon ape leukemia virus (GALV.fus) into the viral genome (15). In either case, the fusogenic oncolytic HSV showed strikingly enhanced antitumor activity, compared with that of the nonfusogenic virus, when tested against locally established liver cancer (15) or lung metastases of breast cancer (14).

[4] The abbreviations used are: EOC, epithelial ovarian cancer; HSV, herpes simplex virus; pfu, plaque-forming unit(s); FBS, fetal bovine serum; BAC, bacterial artificial chromosome; GFP, green fluorescent protein.

We report here the properties of a newer version of the fusogenic oncolytic HSV, in which two membrane fusion mechanisms were incorporated into a single virus (Synco-2D). *In vitro* characterization showed that Synco-2D has a distinctive pattern of syncytial formation, leading to a significant increase in tumor cell killing. When injected directly into the abdominal cavities of mice bearing human ovarian cancer xenografts, Synco-2D induced complete remissions in 75% of the treated animals, in contrast to partial remissions in the group treated with a conventional oncolytic HSV. These results suggest that this newly generated fusogenic oncolytic HSV is a promising candidate for *in vivo* virotherapy against advanced ovarian cancer.

MATERIALS AND METHODS

Cell Lines and Mice. African green monkey kidney cells (Vero) were purchased from American Type Culture Collection (Manassas, VA). The Hey-8 cell line was established from a peritoneal deposit of a moderately differentiated papillary ovarian cystoadenocarcinoma and has been reported to be moderately resistant to chemotherapy (16, 17). SKOv3 cells were derived from serous cystoadenocarcinoma. All cells were cultured in DMEM containing 10% FBS. Female BALB/c mice or Hsd athymic (*nu/nu*) mice [obtained from Harlan (Indianapolis, IN)] were housed under specific pathogen-free conditions and used in experiments when they attained the age of 7–8 weeks.

Construction of Fusogenic HSV Vectors. All oncolytic HSVs were derived from fHSV-Δ-pac, a BAC-based construct that contains a mutated HSV genome, in which the diploid gene encoding γ*34.5* and both copies of HSV packaging signal have been deleted (18). Infectious HSV cannot be generated from this construct unless an intact HSV packaging signal is provided in *cis*; otherwise, the virus will be replication conditional due to the deletion of both copies of γ*34.5*. Baco-1 was constructed by inserting a DNA sequence containing a HSV packaging signal and an enhanced GFP gene cassette into the unique *Pac*I restriction site located in the BAC sequence in fHSV-Δ-pac, as described previously (15). To generate Synco-2D, we initially subjected Baco-1 to random mutagenesis (14). The syncytial phenotype was identified by screening the mutagenized virus on Vero cells. The circular form of viral DNA was then obtained from the new virus (Baco-F1) by extracting virion DNA from Vero cells shortly (1 h) after virus infection, as described previously (19). The viral DNA was then transformed into competent *Escherichia coli* cell DH-10B through electroporation, and Baco-F1 DNA was purified from bacterial growth with the use of a Qiagen kit. To insert the hyperfusogenic glycoprotein gene of gibbon ape leukemia virus (GALV.fus) into Baco-F1, we replaced the gene cassette encoding GFP in Baco-F1 with GALV.fus (driven by the conditional UL38 promoter of HSV) through an enforced ligation strategy (15, 20). The ligation mixture was directly transfected into Vero cells using LipofectAMINE (Life Technologies, Inc.) and incubated for 3–5 days to permit the generation of infectious virus. The resultant viruses were subsequently plaque-purified. Viral stocks were prepared by infecting Vero cells with 0.01 pfu/cell. When cells showed complete cytopathic effect, heparin (Sigma, St. Louis, MO) was added to the culture medium at a final concentration of 50 μg/ml, and cells were cultured for another 3 h to release the virus into the medium. The medium was then collected and subjected to a low-speed centrifugation at 1,000 × *g* for 10 min. The cleared supernatant was transferred to another tube, and the virus was pelleted through high-speed centrifugation (29,000 × *g* for 4 h). The viral pellet was resuspended in PBS containing 10% glycerol and stored at −80°C.

Characterization of Newly Generated HSV Vector and *in Vitro* Cell Killing Assay. For phenotypic characterization of the new virus, Hey-8 or SKOv3 ovarian cancer cells were seeded into 6-well plates and then infected the following day with Baco-1 or Synco-2D at a dose of 0.01 pfu/cell. Cells were cultured in a maintenance medium (containing 1% FBS) and left for up to 2 days to allow the fusion pattern and plaques to develop.

For viral growth characterization, Vero cells seeded in triplicate in 6-well plates were infected with the viruses at 0.01 or 0.1 pfu/cell for 1 h. Cells were washed once with PBS to remove unadsorbed and uninternalized viruses before 1 ml of fresh medium was added. Cells were harvested at 0, 12, 24, 36, and 48 h after infection. Viruses were released by repeated freezing and thawing and sonication. Virus titers were determined on Vero cells by a plaque assay.

To measure *in vitro* killing effect of viruses, Hey-8 or SKOv3 tumor cells were seeded into 24-well plates and infected with Baco-1 or Synco-2D at 0.1 and 0.01 pfu/cell or left uninfected. Cells were harvested 24 or 48 h later through trypsinization. The number of viable cells was counted with a hemocytometer after trypan blue staining. The percentage of viable cells was calculated by dividing the number of cells that excluded trypan blue from the infected well by the number of cells that excluded trypan blue from the well that was left uninfected. The experiments were done in triplicate, and the averaged numbers were used for the final calculation.

Animal Studies. Hey-8 cells were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin and 0.05% EDTA. Trypsinization was stopped with medium containing 10% FBS, and cells were washed once in serum-free medium and resuspended in PBS. Only single cell suspensions with >95% viability were used for *in vivo* injection. Briefly, on day 0, 3×10^5 viable Hey-8 cells were inoculated into the peritoneal cavities of 8-week-old female nude mice. The mice were then randomly divided into three groups of eight mice each. On days 14 and 28 after tumor inoculation, the mice in groups 1 and 2 received i.p. injection with either Baco-1 or Synco-2D at a dose of 2×10^7 pfu in a total volume of 200 μl. Mice in group 3 received the same volume of PBS as the control. The therapeutic injection was given at a site distant from the area of tumor cell inoculation. On day 42 after tumor cell inoculation, all surviving mice were euthanized by CO_2 exposure and evaluated macroscopically for the number and size of tumor nodules in the abdominal cavity. For toxicity evaluation, 7–8-week-old BALB/c mice received injection with two different doses of viruses (5×10^7 or 3×10^8) or the same volume (200 μl) of PBS as a control. There were 5 mice/group. The viruses were prepared with serum-free medium, and the injection was given though the tail vein at a speed of 2 min/injection.

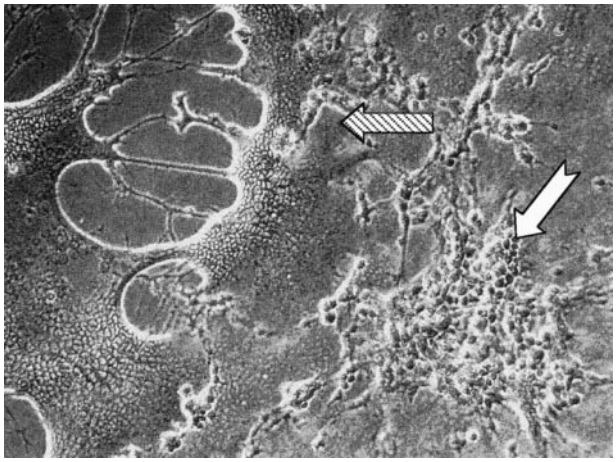

Fig. 1 Phenotypic characterization of Synco-2D in Vero cells. To directly compare the phenotype and plaque size of Baco-1 and Synco-2D, we mixed the two viruses at a 1:1 ratio. The mixture was serially diluted and then used to infect Vero cell monolayers. The photomicrograph, which was taken 40 h after virus infection, shows an area containing plaques from both viruses. The plaque from Synco-2D, in which only a portion is shown, is indicated by the *hatched arrow*; the *open arrow* indicates the plaque from Baco-1. Original magnification, ×200.

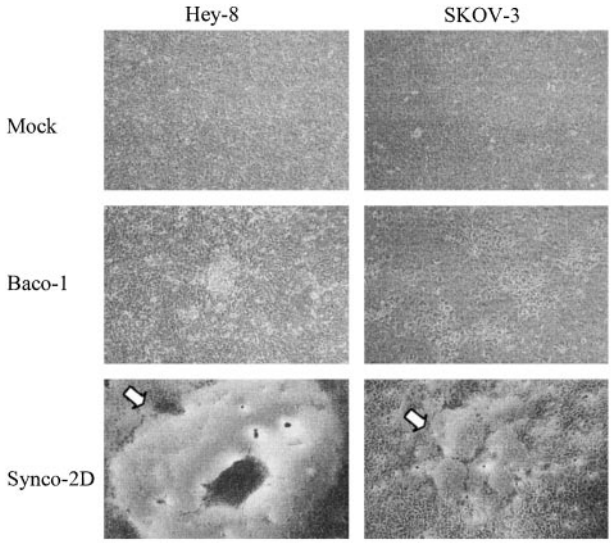

Fig. 2 *In vitro* phenotypic characterization of Synco-2D in ovarian cancer cells. Hey-8 or SKOv3 ovarian cancer cells were infected with either Baco-1 or Synco-2D at 0.001 pfu/cell. Photomicrographs were taken at 48 h after viral infection (original magnification, ×200). *Arrows* indicate a single syncytium.

Statistical Analysis. Quantitative results are reported as means ± SDs. The statistical analysis was performed by Student's *t* test or one-way ANOVA, with the exception of the survival data, which were analyzed by the Kaplan-Meier method and the log-rank (Mantel-Cox) test using Statview 5.0 software (SAS Institute, Inc., Cary, NC). *P*s < 0.05 were considered significant.

RESULTS

Generation and Characterization of Doubly Fusogenic Oncolytic HSV. Membrane fusion capability was first introduced into a conventional oncolytic HSV, Baco-1 (15), through random mutagenesis of incorporation of the thymidine analogue bromodeoxyuridine during viral replication in Vero cells (14). The virus was phenotypically identified and purified to homogeneity. The gene encoding the hyperfusogenic GALV.fus (21), driven by the promoter of the strict-late gene-UL38, was then cloned into the BAC-based viral genome through an enforced ligation strategy (15) to replace the GFP gene of Baco-1. One of the plaque-purified viruses, designated Synco-2D, was chosen for further characterization.

To directly compare the phenotypes and plaque size of Baco-1 and Synco-2D, we mixed the two viruses at a 1:1 ratio, serially diluted, and then used it to infect Vero cell monolayers. Fig. 1 shows a microscopic field containing the plaques from both viruses. The plaque from Synco-2D (*hatched arrow*) was much larger than that of Baco-1 (*open arrow*), so that only a portion of the syncytium could be shown in the photo. The syncytial plaque comprised fused cells with indistinguishable boundaries, whereas the Baco-1 plaque, formed entirely from round cells, was typical of the cytopathic effect produced by conventional HSV infection. The identity of the viruses was confirmed by the presence (Baco-1) or absence (Synco-2D) of GFP expression under the fluorescent microscopy (data not shown).

We phenotypically characterized the viruses in greater detail on two human ovarian cancer cell lines (Hey-8 and SKOv3) infected with either Baco-1 or Synco-2D at 0.001 pfu/cell. As shown in Fig. 2, *bottom panels*, the syncytial plaques after Synco-2D infection differed strikingly from the usual round cell plaques derived from infection with Baco-1. By 40 h, the Synco-2D-infected tumor cells had fused together, forming a dense material (middle of the infectious focus) that was separated from the boundary of the plaque by a large gap area. This distinctive pattern of syncytial development was especially prominent in Hey-8 cells.

We also compared replication properties of Synco-2D with those of Baco-1 and a wild-type HSV (strain 17) on Vero cells. The cells were infected with the viruses at either 0.1 or 0.01 pfu/cell. The viruses were harvested at 12-h intervals after infection and quantified through plaque assay. The results showed that at either dose, the viral titers from Synco-2D had almost 10-fold enhanced ability to replicate relative to Baco-1 in Vero cells (Fig. 3). This result is consistent with our earlier observation on the growth properties of Fu-10, a fusogenic oncolytic HSV selected from G207 through a similar strategy (14). However, the titer of Synco-2D was substantially lower than that of the wild-type HSV strain 17.

Comparison of Tumor Cell Killing *in Vitro*. To determine whether the marked syncytial formation resulting from Synco-2D infection would enhance tumor cell killing, we infected ovarian cancer cells with Baco-1 or Synco-2D at a relatively low multiplicity of infection (0.1 or 0.01 pfu/cell), permitting us to assess both the inherent cytotoxicity of the input virus and the ability of the virus to replicate and spread in these

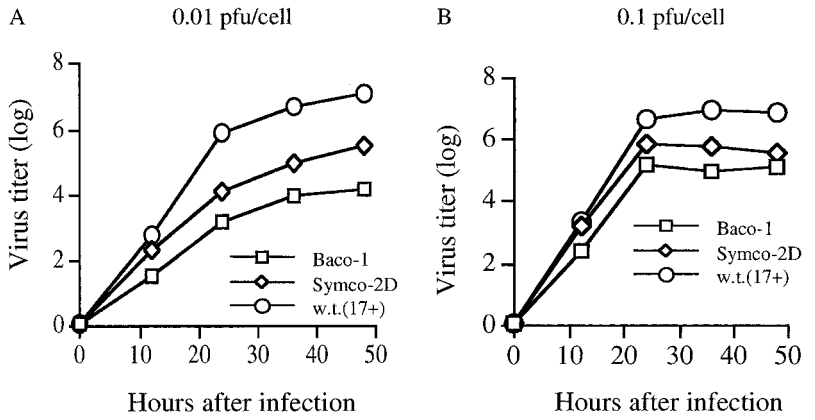

Fig. 3 Replicative properties of Synco-2D. Vero cells were seeded into 6-well plates and infected with the viruses at 0.01 pfu/cell (*A*) or 0.1 pfu/cell (*B*). The viruses were harvested at 0, 12, 24, 36, and 48 h after infection and titrated by plaque assay, and the results were plotted against the time after infection.

cells. The cytotoxic effect of the viruses was quantified by calculating the percentage of viable cells remaining in the wells after infection. The results (Fig. 4) showed a significantly greater tumor cell killing effect by Synco-2D compared with Baco-1 ($P < 0.01$, all comparisons). At 0.01 pfu/cell, Synco-2D reduced the viable cells to <40% within 24 h (Fig. 4*A*). At 0.1 pfu/cell, it completely destroyed the Hey-8 tumor cells within 48 h, a time at which there was still >30% viable tumor cells in the well infected with Baco-1 (Fig. 4*B*). The degree of tumor cell killing appeared to correlate with the extent of syncytial formation (as seen in Fig. 2); that is, both the cytolytic and syncytium-inducing effects of Synco-2D were greater in Hey-8 cells than in SKOv3 cells.

Therapeutic Effects of Synco-2D on Peritoneal Metastases of Ovarian Cancer. Peritoneal invasion of ovarian cancer is a common and serious clinical problem. It has been reported that about 70% of late-stage ovarian cancer patients have metastatic disease in the peritoneal cavity (2). We therefore chose a peritoneal metastasis model (xenografted Hey-8 cells) as a means to test the efficacy of Synco-2D against human ovarian cancer. Freshly harvested Hey-8 cells were inoculated into the peritoneal cavities of nude mice at a dose of 3×10^5 cells/mouse. Two weeks later, palpable tumor had formed near the injection site in all mice. The average tumor diameter was approximately 3 mm. At this time, mice received i.p. injection with 2×10^7 pfu/200 μl of either Baco-1, Synco-2D, or PBS (control) at a site distant from that of tumor cell implantation. A second i.p. injection with the same amount of virus was given 2 weeks later. During the interim period, PBS control mice began to die from tumor or had to be euthanized due to tumor progression and cachexia. Thus, the mean survival time in this group was 36.5 ± 0.7 days (none of the mice survived); there was clear i.p. dissemination of tumor (Fig. 5*A*), with an average of 2.5 ± 0.9 tumor nodules found in regions distal from the site of the tumor implantation (Table 1). In the Baco-1 treatment group, three mice died before the end of the experiment [first death, 34 days after tumor cell implantation (Fig. 6)]; five survivors bore a single large tumor (Fig. 5*B*) when examined at necropsy. By contrast, none of the Synco-2D-treated mice died or were euthanized during the experiment. Strikingly, six of the eight mice were entirely tumor free at necropsy by the end of the experiment (Table 1 and Fig. 5*C*). The other two animals had tumors that are significantly smaller than those in Baco-1-treated mice ($P < 0.01$; Table 1).

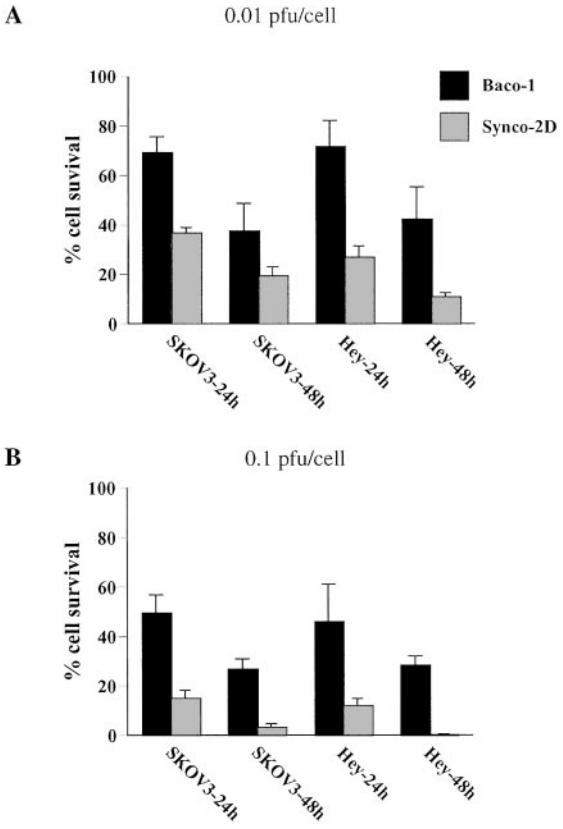

Fig. 4 Comparison of the cytotoxicity of Baco-1 and Synco-2D in cultured ovarian cancer cells. Hey-8 or SKOv3 ovarian cancer cells were seeded into 24-well plates and infected with Baco-1 or Synco-2D at 0.01 (*A*) or 0.1 (*B*) pfu/cell. Cells were collected at 24 or 48 h after infection, stained with trypan blue, and counted. The percentage of cell viability was determined by dividing the number of viable cells from the infected well by the number of cells from an uninfected well. The data are reported as means ± SD. All comparisons showed a significant advantage in cytotoxicity for Synco-2D ($P < 0.01$).

Toxicity Evaluation of Fusogenic Oncolytic HSV. As a first step toward evaluating the toxicity of this doubly fusogenic

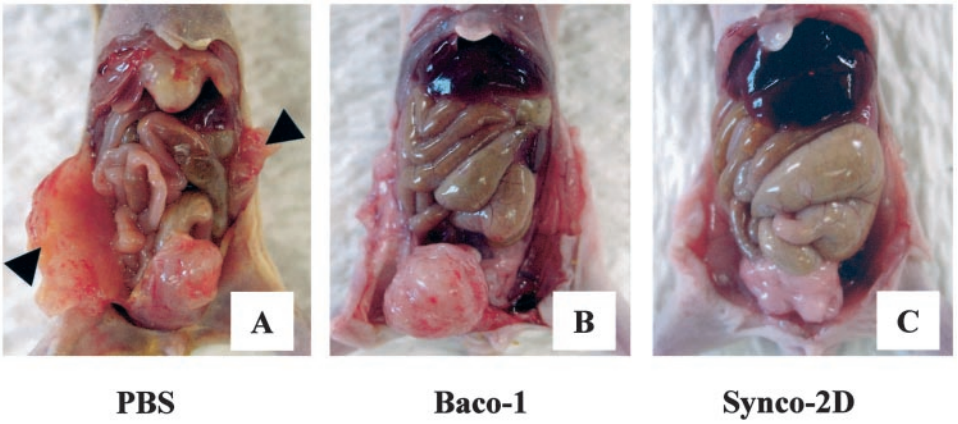

Fig. 5 Effects of Synco-2D in xenograft model of i.p. ovarian cancer. Human ovarian cancer xenografts were established through i.p. injection of 3×10^5 Hey-8 cells into nude mice. The mice then received i.p. injection, on days 14 and 28, with either PBS (control) or 2×10^7 pfu of Baco-1 or Synco-2D. Forty-two days after tumor implantation, any mice that were still alive were euthanized. All mice were examined for the presence of tumor nodules in the abdominal cavity. *A* (PBS), *B* (Baco-1), and *C* (Synco-2D) show the gross pathological findings in a representative mouse from each group.

Table 1 Results of Synco-2D therapy for xenografted human ovarian cancer in the peritoneum

Treatment	*N*	No. of mice with tumors	No. of nodules	Tumor weight (g)[a]	Death rate
PBS	8	8/8	2.5 ± 0.9	1.0 ± 0.6	8/8
Baco-1	8	8/8	1.0 ± 0.0[b]	1.5 ± 0.7	3/8[b]
Synco-2D	8	2/8[c]	0.25 ± 0.4[c]	0.1 ± 0.3[c]	0/8[c]

[a] Means and SDs.
[b] $P < 0.01$ as compared with PBS control.
[c] $P < 0.01$ as compared with either the PBS- or Baco-1-treated group.

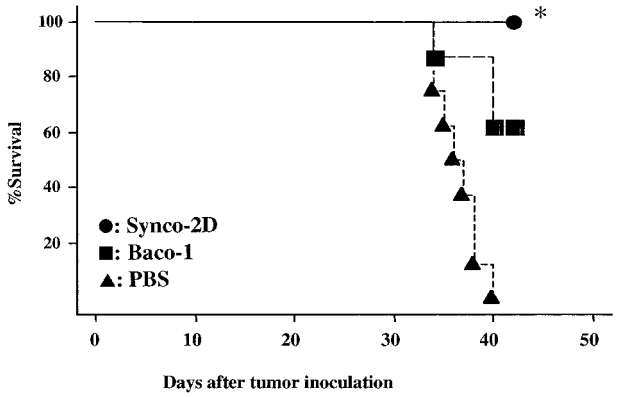

Fig. 6 Prolonged survival of mice treated with Synco-2D. The figure shows the survival curve on Kaplan-Meier plots of data from the experiment described in Fig. 5. The *asterisk* denotes a significant difference between the Synco-2D group and either the PBS or Baco-1 group ($P < 0.01$).

oncolytic HSV, we injected either Baco-1 or Synco-2D at relatively high doses (5×10^7 and 3×10^8 pfu) into immunocompetent BALB/c mice through the tail vein ($n = 5$ mice/dose). The dose of 3×10^8 pfu in 200 μl of solution is the maximum that could be injected systemically into mice, given the modest scale of virus preparation (maximum titer, $<2 \times 10^9$ pfu/ml). The mice were then observed for 6 weeks. Shortly after virus injection, two mice receiving the higher dose of Baco-1 became inactive, but they recovered in less than 3 h. Otherwise, there were no animal deaths or evidence of disease during the observation period. These results indicate that, despite its high antitumor potency, Synco-2D is probably not much more toxic than its parental nonfusogenic virus.

DISCUSSION

In our previous study, we had shown that a syncytial mutant (Fu-10) selected from the well-characterized oncolytic G207 virus through random mutagenesis had potent antitumor activity against lung metastases of breast cancer (14). We also showed that insertion of the hyperfusogenic glycoprotein from gibbon ape leukemia virus into a conventional oncolytic HSV can significantly increase the antitumor potency of that virus (15). Here, we created Synco-2D, a modified version of the fusogenic oncolytic HSV, by incorporating both fusion mechanisms into a single oncolytic HSV. *In vitro* characterization studies showed that infection with Synco-2D promotes extremely strong syncytial formation in both Vero and human ovarian tumor cell lines. The i.p. administration of this virus to animals bearing established human ovarian cancer in the abdominal cavity produced striking therapeutic effects: six of eight mice became totally tumor free, in contrast to the presence of bulky tumors in all mice treated with the conventional oncolytic virus. This result suggests that strategies similar to ours would significantly increase the likelihood of success in clinical trails of oncolytic HSVs in patients with advanced ovarian cancer and perhaps other malignant solid tumors as well.

Although we did not directly compare the doubly fusogenic virus with either of the two previously described HSVs with a single membrane fusion capability (14, 15), syncytial formation was clearly greater with Synco-2D, suggesting enhancement of antitumor activity. Additionally, virotherapy with Synco-2D

should reduce the emergence of treatment resistance. This is because syncytial formation mediated by fusogenic glycoproteins relies on the initial binding of fusogenic glycoproteins with their specific receptors on target cells, which then induces ordered structural changes of the membrane lipid bilayers, leading in turn to lipid mixing and eventually to membrane fusion either between viral and cellular membranes or among cellular membranes (22). The envelope glycoprotein of GALV is the only viral protein required for both viral and cellular membrane fusion. The cellular receptor for GALV has been identified as Pit1, a type III sodium-dependent phosphate transporter (23, 24). The membrane fusion induced by HSV is more complex, requiring the participation of multiple viral glycoproteins and at least two specific receptors on the cell surface, heparan sulfate and the newly discovered HVEM [herpesvirus entry mediator (25, 26)]. Therefore, it is possible that tumor cells that become resistant to one fusion protein (*e.g.*, due to the lack of a requisite cellular receptor) will still be destroyed by syncytial formation resulting from expression of the other fusion protein, which uses a totally different cellular receptor to initiate the membrane fusion process. Our recent finding that infection with Synco-2D but not with Fu-10 or Synco-2 can cause syncytia formation in several murine tumor cell lines and one human tumor line supports this possibility.[5]

The peritoneal xenograft model of human ovarian cancer used to evaluate the efficacy of Synco-2D has definite clinical relevance. Although patients with stage I EOC (localized to ovaries) have a 5-year survival rate of 90% after surgical resection (27), those with tumor spreading beyond the ovaries, often to the peritoneal cavity, usually have very poor prognosis. Because the disease frequently remains confined to the peritoneal surfaces in these patients (28), successful management of peritoneal metastases can directly improve prognosis. There are also several additional advantages to administering oncolytic virus i.p. The peritoneal cavity serves as a restricted compartment, allowing maximal contact and interaction between cancer cells and therapeutic agents. It also circumvents encounters with neutralizing antibodies and reduces toxicity by comparison with systemic administration. To closely mimic clinical situations, we administered the oncolytic viruses at a relatively late stage after tumor cell implantation, when all of the mice had tumors that were palpable through the abdominal wall. The viral dose of 2×10^7 pfu, which eradicated tumor in 75% of the mice, is equivalent to 6×10^{10} pfu in humans, a dose similar to the highest dose of G207 administered in a Phase I study for malignant gliomas (10). Bearing in mind that the peritoneal cavity represents a substantially larger area than the brain and is probably less vulnerable to any potential toxic effects of the virus, we believe that such a virus dose should be safe and well tolerated in patients. Thus, direct administration of Synco-2D, alone or in association with a surgical debulking procedure to remove gross recurrent disease, is a reasonable strategy for clinical testing.

Uncontrolled induction of syncytial formation by fusogenic oncolytic HSVs is a potential safety concern. We believe that both of the fusion mechanisms in Synco-2D are tumor specific. First, the syncytial formation from mutagenized HSV is mainly due to aberrant expression of several viral glycoproteins, such as gB and gK (29–32). Because these glycoproteins are encoded by late genes whose expression depends upon viral DNA replication, such oncolytic viruses will retain the safety of the original virus because syncytial formation will only occur in tumor cells (where virus can undergo a full infection cycle) and not in normal nondividing cells (where virus replication is restricted, and very low levels of glycoproteins are expressed). To control the fusogenic activity from GALV.fus, we used a strict late viral promoter to direct gene expression, the activity of which has been shown to remain confined to the tumor tissue after systemic administration with an oncolytic HSV (20). Thus, GALV.fus-mediated syncytial formation was linked to the virus to replicate conditionally in tumor cells. The demonstrations in our previous studies that blocking viral DNA replication completely abolishes syncytia-forming ability of Fu-10 (the selected syncytial mutant from G207; Ref. 14) and that Synco-2 (containing GALV.fus driven by UL38p) cannot induce syncytial formation in nondividing cells (15) strongly suggest that Synco-2D retains the safety profile of a conventional oncolytic HSV. This notion is supported by the observation that i.v. injection of Synco-2D in relatively large doses (up to 3×10^8 pfu) is well tolerated in mice.

[5] Unpublished observation.

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REFERENCES

1. Greenlee, R. T., Hill-Harmon, M. B., Murray, T., and Thun, M. Cancer statistics, 2001. CA Cancer J. Clin. *51*: 15–36, 2001.

2. Buy, J. N., Moss, A. A., Ghossain, M. A., Sciot, C., Malbec, L., Vadrot, D., Paniel, B. J., and Decroix, Y. Peritoneal implants from ovarian tumors: CT findings. Radiology, *169*: 691–694, 1988.

3. Ozols, R. F. Management of advanced ovarian cancer consensus summary. Advanced Ovarian Cancer Consensus Faculty. Semin. Oncol. *27*: 47–49, 2000.

4. Martuza, R. L., Malick, A., Markert, J. M., Ruffner, K. L., and Coen, D. M. Experimental therapy of human glioma by means of a genetically engineered virus mutant. Science (Wash. DC), *252*: 854–856, 1991.

5. Mineta, T., Rabkin, S. D., Yazaki, T., Hunter, W. D., and Martuza, R. L. Attenuated multi-mutated herpes simplex virus-1 for the treatment of malignant gliomas. Nat. Med., *1:* 938–943, 1995.

6. Bolovan, C. A., Sawtell, N. M., and Thompson, R. L. ICP34.5 mutants of herpes simplex virus type 1 strain 17syn+ are attenuated for neurovirulence in mice and for replication in confluent primary mouse embryo cell cultures. J. Virol., *68*: 48–55, 1994.

7. Chou, J., and Roizman, B. The γ 1(34.5) gene of herpes simplex virus 1 precludes neuroblastoma cells from triggering total shutoff of protein synthesis characteristic of programmed cell death in neuronal cells. Proc. Natl. Acad. Sci. USA, *89*: 3266–3270, 1992.

8. McKie, E. A., MacLean, A. R., Lewis, A. D., Cruickshank, G., Rampling, R., Barnett, S. C., Kennedy, P. G., and Brown, S. M. Selective *in vitro* replication of herpes simplex virus type 1 (HSV-1) ICP34.5 null mutants in primary human CNS tumours: evaluation of a potentially effective clinical therapy. Br. J. Cancer, *74:* 745–752, 1996.

9. Rampling, R., Cruickshank, G., Papanastassiou, V., Nicoll, J., Hadley, D., Brennan, D., Petty, R., MacLean, A., Harland, J., McKie, E., Mabbs, R., and Brown, M. Toxicity evaluation of replication-competent

herpes simplex virus (ICP 34.5 null mutant 1716) in patients with recurrent malignant glioma. Gene Ther., *7*: 859–866, 2000.

10. Markert, J. M., Medlock, M. D., Rabkin, S. D., Gillespie, G. Y., Todo, T., Hunter, W. D., Palmer, C. A., Feigenbaum, F., Tornatore, C., Tufaro, F., and Martuza, R. L. Conditionally replicating herpes simplex virus mutant, G207 for the treatment of malignant glioma: results of a Phase I trial. Gene Ther., *7*: 867–874, 2000.

11. Kramm, C. M., Chase, M., Herrlinger, U., Jacobs, A., Pechan, P. A., Rainov, N. G., Sena-Esteves, M., Aghi, M., Barnett, F. H., Chiocca, E. A., and Breakefield, X. O. Therapeutic efficiency and safety of a second-generation replication-conditional HSV1 vector for brain tumor gene therapy. Hum. Gene Ther., *8:* 2057–2068, 1997.

12. Todo, T., Martuza, R. L., Rabkin, S. D., and Johnson, P. A. Oncolytic herpes simplex virus vector with enhanced MHC class I presentation and tumor cell killing. Proc. Natl. Acad. Sci. USA, *98*: 6396–6401, 2001.

13. Andreansky, S., Soroceanu, L., Flotte, E. R., Chou, J., Markert, J. M., Gillespie, G. Y., Roizman, B., and Whitley, R. J. Evaluation of genetically engineered herpes simplex viruses as oncolytic agents for human malignant brain tumors. Cancer Res., *57:* 1502–1509, 1997.

14. Fu, X., and Zhang, X. Potent systemic antitumor activity from an oncolytic herpes simplex virus of syncytial phenotype. Cancer Res., *62:* 2306–2312, 2002.

15. Fu, X., Tao, L., Jin, A., Vile, R., Brenner, M., and Zhang, X. Expression of a fusogenic membrane glycoprotein by an oncolytic herpes simplex virus provides potent synergistic anti-tumor effect. Mol. Ther., in press, 2003.

16. Selby, P. J., Thomas, J. M., Monaghan, P., Sloane, J., and Peckham, M. J. Human tumour xenografts established and serially transplanted in mice immunologically deprived by thymectomy, cytosine arabinoside and whole-body irradiation. Br. J. Cancer, *41*: 52–61, 1980.

17. Buick, R. N., Pullano, R., and Trent, J. M. Comparative properties of five human ovarian adenocarcinoma cell lines. Cancer Res., *45*: 3668–3676, 1985.

18. Saeki, Y., Ichikawa, T., Saeki, A., Chiocca, E. A., Tobler, K., Ackermann, M., Breakefield, X. O., and Fraefel, C. Herpes simplex virus type 1 DNA amplified as bacterial artificial chromosome in *Escherichia coli*: rescue of replication-competent virus progeny and packaging of amplicon vectors. Hum. Gene Ther., *9*: 2787–2794, 1998.

19. Zhang, X., Efstathiou, S., and Simmons, A. Identification of novel herpes simplex virus replicative intermediates by field inversion gel electrophoresis: implications for viral DNA amplification strategies. Virology, *202:* 530–539, 1994.

20. Fu, X., Meng, F., Tao, L., Jin, A., and Zhang, X. A strict late viral promoter is a strong tumor-specific promoter in the context of an oncolytic herpes simplex virus. Gene Ther., in press, 2003.

21. Fielding, A. K., Chapel-Fernandes, S., Chadwick, M. P., Bullough, F. J., Cosset, F. L., and Russell, S. J. A hyperfusogenic gibbon ape leukemia envelope glycoprotein: targeting of a cytotoxic gene by ligand display. Hum. Gene Ther., *11*: 817–826, 2000.

22. Lentz, B. R., Malinin, V., Haque, M. E., and Evans, K. Protein machines and lipid assemblies: current views of cell membrane fusion. Curr. Opin. Struct. Biol., *10*: 607–615, 2000.

23. Johann, S. V., van Zeijl, M., Cekleniak, J., and O'Hara, B. Definition of a domain of GLVR1 which is necessary for infection by gibbon ape leukemia virus and which is highly polymorphic between species. J. Virol., *67*: 6733–6736, 1993.

24. Kavanaugh, M. P., Miller, D. G., Zhang, W., Law, W., Kozak, S. L., Kabat, D., and Miller, A. D. Cell-surface receptors for gibbon ape leukemia virus and amphotropic murine retrovirus are inducible sodium-dependent phosphate symporters. Proc. Natl. Acad. Sci. USA, *91*: 7071–7075, 1994.

25. Terry-Allison, T., Montgomery, R. I., Warner, M. S., Geraghty, R. J., and Spear, P. G. Contributions of gD receptors and glycosaminoglycan sulfation to cell fusion mediated by herpes simplex virus 1. Virus Res., *74*: 39–45, 2001.

26. Terry-Allison, T., Montgomery, R. I., Whitbeck, J. C., Xu, R., Cohen, G. H., Eisenberg, R. J., and Spear, P. G. HveA (herpesvirus entry mediator A), a coreceptor for herpes simplex virus entry, also participates in virus-induced cell fusion. J. Virol., *72*: 5802–5810, 1998.

27. Rubin, S. C., Randall, T. C., Armstrong, K. A., Chi, D. S., and Hoskins, W. J. Ten-year follow-up of ovarian cancer patients after second-look laparotomy with negative findings. Obstet. Gynecol., *93*: 21–24, 1999.

28. Pickel, H., Lahousen, M., Girardi, F., Tamussino, K., and Stettner, H. Intraperitoneal and retroperitoneal spread of ovarian cancer. *In:* F. Sharp, W. P. Mason, and R. E. Leake (eds.), Ovarian Cancer: Biological and Therapeutic Challenges, pp. 171–178. London: Chapman & Hall Medical, 1989.

29. Read, G. S., Person, S., and Keller, P. M. Genetic studies of cell fusion induced by herpes simplex virus type 1. J. Virol., *35*: 105–113, 1980.

30. Bond, V. C., Person, S., and Warner, S. C. The isolation and characterization of mutants of herpes simplex virus type 1 that induce cell fusion. J. Gen. Virol., *61*: 245–254, 1982.

31. Pogue-Geile, K. L., Lee, G. T., Shapira, S. K., and Spear, P. G. Fine mapping of mutations in the fusion-inducing MP strain of herpes simplex virus type 1. Virology, *136*: 100–109, 1984.

32. Person, S., Kousoulas, K. G., Knowles, R. W., Read, G. S., Holland, T. C., Keller, P. M., and Warner, S. C. Glycoprotein processing in mutants of HSV-1 that induce cell fusion. Virology, *117*: 293–306, 1982.

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ORIGINAL ARTICLE

An oncolytic virus derived from type 2 herpes simplex virus has potent therapeutic effect against metastatic ovarian cancer

X Fu[1,2], L Tao[1,2] and X Zhang[1,2,3]

[1]*Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX, USA;* [2]*Department of Pediatrics, Baylor College of Medicine, Houston, TX, USA and* [3]*Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, TX, USA*

Oncolytic viruses derived from herpes simplex virus (HSV) have shown considerable promise as antitumor agents against solid tumors including ovarian cancer. The current group of oncolytic HSVs was constructed exclusively from type 1 HSV. To exploit further the therapeutic potential of replication-selective viruses, we constructed an oncolytic virus from type 2 HSV by deleting the protein kinase domain of the viral *ICP10* gene, which targets the activated Ras signaling pathway in tumor cells. In the study reported here, we administered this HSV-2-derived virus intraperitoneally (i.p.) to nude mice bearing metastatic human ovarian tumor xenografts, evaluated its oncolytic activity, and compared with to that of a virus constructed from HSV-1. Two injections of the HSV-2-derived virus (3×10^6 pfu per dose) led to complete eradication of disseminated tumors in the peritoneal cavity in more than 87% of the mice, whereas the HSV-1-based oncolytic virus, administered at the same dose and on the same schedule, eradicated tumor nodules in only 12% of mice ($P<0.01$). We conclude that i.p. administration of this HSV-2-based oncolytic virus may provide effective treatment for metastatic human ovarian cancer.
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Keywords: oncolytic virus; HSV-2; ovarian cancer; metastatic; Ras signaling pathway

Introduction

Ovarian cancer remains the leading cause of death in patients with gynecological malignancies in the United States. Because of ineffective screening strategies and inconspicuous early symptoms, these tumors are usually detected late in the clinical course, when metastases are present. The primary site of metastatic spread is within the peritoneal cavity. Despite improved surgical techniques and chemotherapy, the mortality rate for ovarian cancer has remained largely unchanged over the last decade,[1,2] mandating the development of new treatment strategies.

Replication-selective viruses afford a promising treatment for malignant solid tumors. Oncolytic viruses derived from herpes simplex virus (HSV) were initially designed and constructed for the treatment of brain tumors.[3,4] Subsequently, they have proved effective against a variety of other human solid tumors, including ovarian cancer.[5,6] The current group of oncolytic HSVs were constructed exclusively from type 1 virus (HSV-1), most often by deleting the *γ34.5* gene, which encodes a neurovirulent factor, and/or insertional mutation of the *ICP6* gene, which encodes the large subunit of ribonucleotide reductase.[3,4,7–9] Inactivation of either or both of these genes enables the virus to replicate selectively in dividing cells whereas sparing normal *nondividing cells.[10–12]

We have recently constructed a new oncolytic virus from type 2 HSV (HSV-2) to exploit a unique feature of the viral *ICP10* gene, which contains in its N terminus a well-defined region that encodes a serine/threonine protein kinase (PK) activity.[13] As this PK domain can bind and phosphorylate the GTPase-activating protein Ras-GAP, which leads to activation of the Ras/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (MEK)/MAPK mitogenic pathway and c-Fos induction and stabilization, it is required for efficient HSV-2 replication.[14,15] A mutant HSV-2 virus (FusOn-H2), deleted for this PK domain, replicated selectively in and thus lysed human tumor cells. Infection with this virus also induced syncytia formation in tumor cells, providing an additional oncolytic mechanism that enhanced its antitumor effect overall.[16]

Here, we report the therapeutic effects of FusOn-H2 against metastatic human ovarian cancer xenografts growing in the peritoneal cavity of nude mice. Our results indicate that this mutant virus may be an effective oncolytic agent in the treatment of metastatic

Correspondence: Dr X Zhang, Center for Cell and Gene Therapy, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA.
E-mail: xzhang@bcm.tmc.edu
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human ovarian cancer. Indeed, two intraperitoneal (i.p.) injections of the virus at a moderate dose completely eradicated disseminated tumors in the peritoneal cavity of most animals, in contrast to the weak activity of a conventional oncolytic virus derived from type 1 HSV.

Materials and methods

Cell lines and viruses

African green monkey kidney (Vero) cells were obtained from American Type Culture Collection (Rockville, MD). SKOV3 cells derived from a serous cystoadenocarcinoma were kindly provided by Dr Robert Bast (M D Anderson Cancer Center). All cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS). Female Hsd athymic (nu/nu) mice (obtained from Harlan, Indianapolis, Indiana) were kept under specific pathogen-free conditions and used in experiments when they attained the age of 7–8 weeks.

Construction of the HSV-1-derived oncolytic virus Baco-1 is described elsewhere,[6,17] as is construction of FusOn-H2.[16] Briefly, Baco-1 was constructed from a bacterial artificial chromosome (BAC)-based construct that contains a mutated HSV genome, in which the diploid gene encoding *γ34.5* and both copies of the HSV packaging signal (pac) have been deleted.[18] The green fluorescent protein (GFP) gene driven by the cytomegalovirus (CMV) promoter, together with a copy of the HSV-1 pac, were inserted into the unique *PacI* site of fHSV-delta-pac by direct ligation, to generate the infectious HSV (Baco-1). Thus, Baco-1 has both copies of the *γ34.5* gene deleted and contains the GFP marker gene.[17]

For construction of FusOn-H2, the *ICP10* left-flanking region of the wild-type (wt) HSV-2 strain 186 (wt186) genome (equivalent to nucleotide span 85994–86999 in the HSV-2 genome), the ribonucleotide reductase domain and the right-flanking region (equivalent to nucleotide span 88228–89347) were amplified by PCR. These PCR products were cloned together into pNeb193, generating pNeb-ICP10-deltaPK. Hence, the new plasmid contains a mutated *ICP10* gene, in which the protein kinase domain (equivalent to nucleotide span 86999–88228) is deleted. Then, the DNA sequence containing the CMV promoter-*EGFP* gene was PCR amplified from pSZ-EGFP, and the PCR-amplified DNA was cloned into the deleted *PK* locus of pNeb-ICP10-deltaPK, generating pNeb-PKF-2. During the design of PCR-amplification strategies, the *EGFP* gene was fused in-frame with the remaining RR domain of the *ICP10* gene, so that the new protein product of this gene contained the intact EGFP. The modified *ICP10* gene was inserted into the genome of wt186 by homologous recombination. The recombinant virus (FusOn-H2) was identified by screening for GFP-positive plaques. Viral stocks were prepared by infecting Vero cells with 0.01 plaque-forming units (pfu) per cell, harvesting the virus after 2 days, and storing it at −80°C.

Phenotypic characterization and oncolytic activity of FusOn-H2 against a human ovarian cancer cell line

To phenotypically characterize the new virus, we seeded SKOV3 ovarian cancer cells into six-well plates, and then infected them on the following day with Baco-1, wt186 or FusOn-H2 at a dose of 0.01 pfu/cell. Cells were cultured in a maintenance medium (containing 1% FBS) and were incubated for up to 2 days to allow the fusion pattern and plaques to develop. To measure the *in vitro* killing effect of the viruses, we seeded SKOV3 tumor cell lines into 24-well plates and infected them with Baco-1 or FusOn-H2 at 0.01 and 0.001 pfu/cell, or left them uninfected. Cells were harvested 24, 48 or 72 h later by trypsinization, and the number of viable cells determined with a hemocytometer after Trypan blue staining. The percentage of viable cells was calculated by dividing the number of cells excluding Trypan blue in the infected well by the number excluding the stain in the well that was left uninfected. The experiments were performed in triplicate, with mean cell numbers used for the final calculation.

Animal studies

SKOV3 cells were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin and 0.05% ethylenediaminetetraacetic acid. Trypsinization was stopped with medium containing 10% FBS, and the cells were washed once in serum-free medium and resuspended in phosphate-buffer saline (PBS). Only single cell suspensions with >95% viability were used for *in vivo* injection. Briefly, on day 0, 2×10^6 SKOV3 cells were inoculated into the peritoneal cavities of 8-week-old female nude mice. The mice were then randomly divided into three groups of eight mice each. On days 8 and 15 after tumor inoculation, the mice in groups 1 and 2 were injected i.p. with either Baco-1 or FusOn-H2 at a dose of 3×10^6 pfu in a total volume of 200 μl. Mice in group 3 received the same volume of PBS as the control. The therapeutic injection was given at a site distant from the area of tumor cell inoculation. On day 36 after tumor cell inoculation, all surviving mice were euthanized by CO_2 exposure and evaluated macroscopically for the number and size of tumor nodules in the abdominal cavity.

Systemic and intracerebral virus injections

For systemic administration, the virus was diluted in 100 μl of serum-free medium and injected into the animal through the tail-vein as described.[19] Intracranial injection was performed essentially as described.[20] Briefly, mice were anesthetized and placed in a stereotactic frame (Stoelting). A bur hole was drilled in the skull 1 mm anterior and 2 mm lateral to the bregma with a 0.9 mm burr to expose the dura. The mice were injected with 3 μl of serum-free medium containing the virus, by use of a 100 μl syringe (Hamilton) fitted with a 26-gauge needle and connected to the manipulating arm of the stereotactic frame. The injection was directed to the caudate nucleus at a depth of 3.5 mm from the dura and given over 2.5 min. The needle was left in place for 3 min and then withdrawn slowly over another minute, to prevent reflux of the virus solution.

Immunohistochemical staining for HSV antigens in liver sections

Livers were removed 3 or 4 days after systemic administration of HSVs, fixed with 4% paraformaldehyde overnight at 4°C, and treated with 25% sucrose overnight at 4°C. Ten micrometer sections were then prepared, air-dried at room temperature overnight and treated with 4% paraformaldehyde for 2 min, before being treated with 1% NP40/PBS for 5 min. This procedure was followed by several washes with PBS. The liver sections were then incubated with a rat anti-HSV serum (prepared by our own lab at a 1:100 dilution), at 37°C for 60 min. After washing, fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG antibody (Sigma-Aldrich, St Louis, MO), diluted at 1:200, was added to the tissue section and incubated at 37°C for 60 min. After two washes with PBS, the slides were dehydrated in an ascending series of ethanol for 2 min, cleared in xylene for 2 min, mounted in DPX mounting solution, and observed under a fluorescence microscope.

Statistical analysis

Quantitative results are reported as means $\pm$ s.d. The statistical analysis was performed by Student's *t*-test or one-way ANOVA. *P*-values of less than 0.05 were considered significant.

Results

Growth property and phenotype of FusOn-H2 in human ovarian cancer cells

We initially compared the growth of FusOn-H2 with that of its parental wt186 virus and Baco-1. Cells seeded in six-well plates were infected with the viruses at a relatively low dose (0.01 pfu/cell) and harvested at 48 h post-infection, enabling us to assess both the inherent infectivity of the input virus and its ability to replicate and spread in these cells. The results showed that FusOn-H2 replicated well in SKOV3 cells, reaching a titer of almost 1×10^5 pfu/ml (Figure 1). Overall, the titer was about 1 log lower than that of the wt186 or Baco-1 virus, most likely because of the induction of apoptosis in FusOn-H2-infected cells (Fu *et al*., unpublished data).

FusOn-H2 has a fusogenic phenotype in the human breast cancer cell line MDA-MB-435.[16] To determine if it retains this phenotype in human ovarian cancer cells, we infected SKOV3 cells in a six-well plate with either the parental wt186 or FusOn-H2 virus, and monitored the cultures for cytopathic effects and syncytia formation. At 24 h postinfection (Figure 2), syncytia formation was clearly present in cultures infected with FusOn-H2, whereas neither the parental wt186 nor Baco-1 virus produced fusogenic phenotype. Instead, they induced a typical cytopathic effect, represented by cell rounding and swelling. At 48 h after infection, most of the SKOV3 cells infected with FusOn-H2 had detached from the plate, leaving fusion bodies and other cell debris floating in the medium. There was some minimal syncytia formation in the well infected with wt186, but the overall effect differed markedly from that of FusOn-H2 observed at either 24 or 48 h postinfection. At the later stage of infection, the

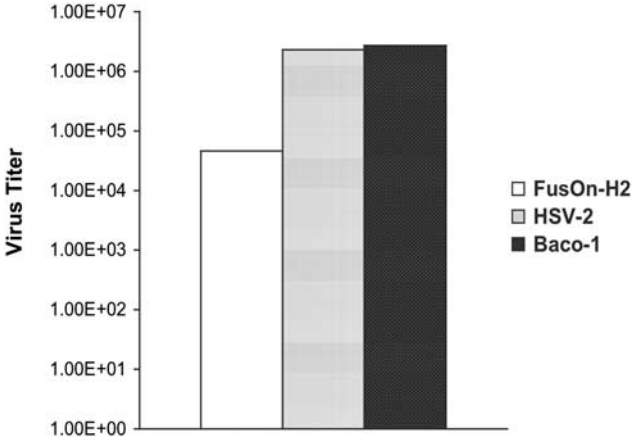

Figure 1 Comparison of the replicative properties of FusOn-H2, Baco-1 and wt HSV-2 in SKOV3 cells. SKOV3 cells were seeded into 24-well plates in triplicate and infected with the viruses at 0.01 pfu/cell. The viruses were harvested at 48 h after infection and titrated by plaque assay on Vero cell monolayers.

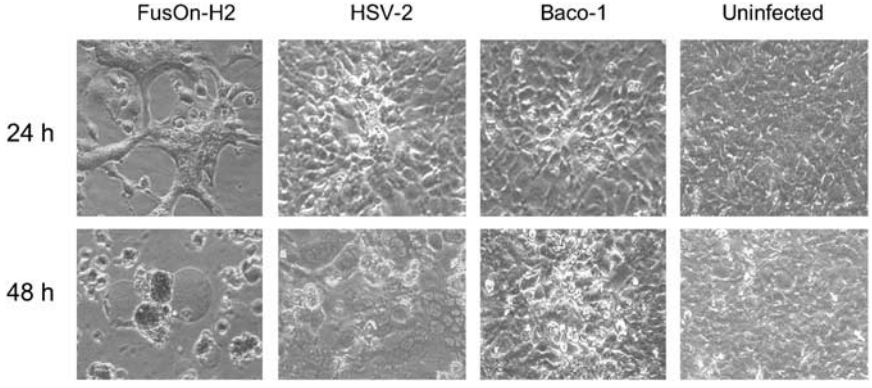

Figure 2 Phenotypic characterization of FusOn-H2 in SKOV3 cells. SKOV3 cell monolayers were infected with the indicated viruses at 0.01 pfu/cell. Micrographs were taken at either 24 or 48 h after infection (original magnification $\times$ 200).

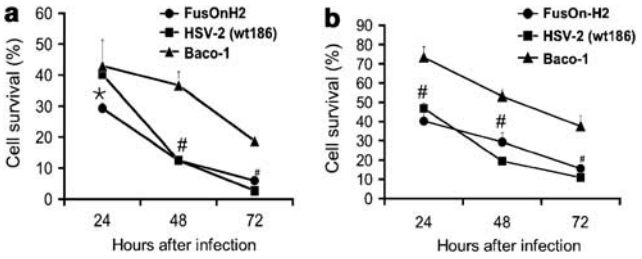

Figure 3 Oncolytic activity of FusOn-H2 against SKOV3 cells *in vitro*. SKOV3 cells were seeded into 24-well plates and infected with FusOn-H2, Baco-1, or the wt HSV-2 virus wt186 at 0.01 pfu/cell (**a**) or 0.001 pfu/cell (**b**), or left uninfected (not shown). Cells were collected 24, 48 or 72 h after infection, and viable cells were counted after Trypan blue staining. The percent cell viability was determined by dividing the number of viable cells in the infected wells by the number of cells in the uninfected well. The data are reported as means $\pm$ s.d. $^{*}P<0.05$ as compared with Baco-1 and wt186; $^{\#}P<0.05$ as compared with Baco-1.

plaques in the well infected with Baco-1 virus were becoming larger, but there were still no sign of syncytia formation (Figure 2).

Efficiency of FusOn-H2-induced killing of human ovarian cancer cells in vitro

To assess the cytolytic activity of FusOn-H2, we infected SKOV3 cells seeded in 24-well plates with this virus, wt186 and Baco-1 at a dose of either 0.01 or 0.001 pfu/cell. The cells were harvested at different times after infection, and cell viability was determined by Trypan blue staining. At 0.01 pfu/cell (Figure 3a), infection with FusOn-H2 killed significantly more tumor cells by 24 h postinfection than did either wt186 or Baco-1 ($P<0.05$). The extend of FusOn-H2-induced killing was similar to that of wt186 at 48 and 72 h postinfection, but remained significantly greater than that of Baco-1 at these two later time points ($P<0.05$). At 0.001 pfu/cell (Figure 3b), the oncolytic effect of FusOn-H2 was equivalent to that of wt186 at all the time points, whereas both viruses were significantly more oncolytic than Baco-1 ($P<0.05$). We also evaluated the *in vitro* oncolytic activity of FusOn-H2 against another human ovarian cancer line, Hey8. Overall, the antitumor effect was essentially the same as obtained with SKOV3 cells (data not shown).

Evaluation of FusOn-H2 oncolytic activity against metastatic ovarian cancer

We chose a peritoneal metastasis model to evaluate the antitumor effects of FusOn-H2 against ovarian cancer. This model is clinically relevant, as peritoneal invasion by ovarian cancer is common and usually represents a serious complication. Freshly harvested SKOV3 cells were inoculated into the peritoneal cavities of nude mice at 2×10^6 cells/mouse. Eight days later, the mice were i.p. injected with 3×10^6 pfu of either Baco-1 or FusOn-H2 or PBS (control) at a site distant from that of tumor cell implantation. At the time of injection, animals lacked any sign of ascites, but they had either visible or microscopic tumors in the peritoneal cavity.

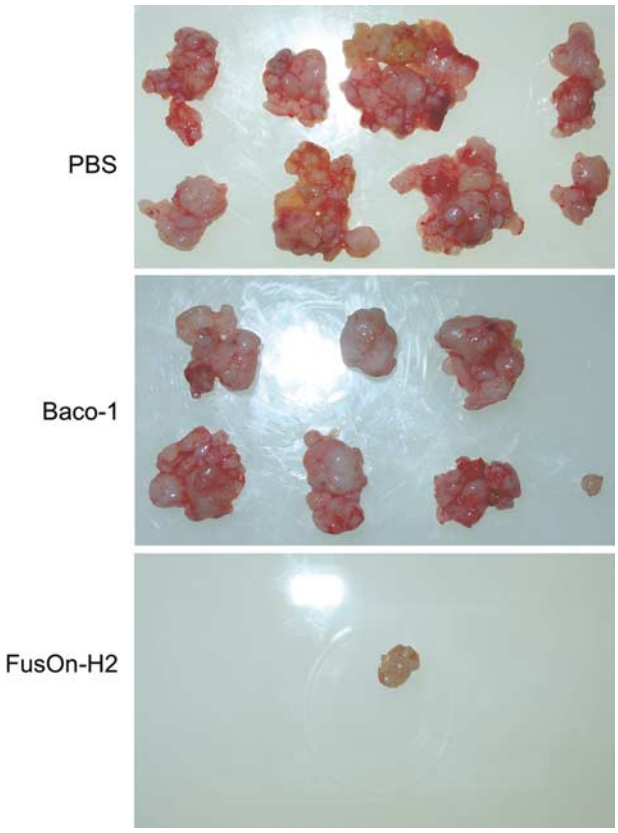

Figure 4 Therapeutic effect of FusOn-H2 against metastatic human ovarian cancer xenografts established in the peritoneal cavity of nude mice. Human ovarian cancer xenografts were established by i.p. inoculation of 2×10^6 SKOV3 cells into the peritoneal cavity ($n=$ eight mice per treatment group). Eight and 15 days after tumor cell inoculation, the mice received an i.p. injection of oncolytic HSVs at a dose of 3×10^6 pfu, at a site distant from the tumor implantation site. Four weeks after the initial virus injection (i.e., 5 weeks after tumor cell implantation), the mice were euthanized. Tumor nodules in the abdomen cavity were examined, counted and excised. The photos show the gross appearance of the excised tumor nodules.

This injection was repeated at the same dose 1 week later. Results from the PBS control group (Figure 4, PBS) showed that implantation of these tumor cells in the specified quantify leads to the formation of disseminated metastatic tumor nodules. On average, there were more than 15 tumor nodules in each mouse, and the tumors from each mouse weighed more than 1 g. Direct injection of FusOn-H2 into the peritoneal cavity produced a highly significant therapeutic effect, completely eradicating all tumor nodules in seven of eight mice (Figure 4, FusOn-H2). The one mouse that was not tumor-free had only a single nodule. Although Baco-1 showed significant oncolytic activity by comparison with the PBS control (Figure 4, also Table 1, $P<0.01$), it was clearly less effective than FusOn-H2, in terms of both the percentage of tumor-free animals (12.5 vs 87.5%, $P<0.01$) and the weight of the remaining tumor nodules (0.7 ± 0.4 g vs 0.02 ± 0.05 g, $P<0.01$). These results clearly demonstrate the ability of FusOn-H2 to eradicate or control metastatic

ovarian cancer established in the peritoneal cavity of mice, even when it is administered with limited frequency and in a very moderate dose.

FusOn-H2 toxicity in vivo

Our previous data showed that FusOn-H2 could not replicate in normal nondividing cells such as primary hepatocytes,[16] indicating that it is probably a safe oncolytic agent for *in vivo* use. To assess its toxicity *in vivo* more extensively, we injected the virus systemically (via the tail vein) into immune-competent Balb/c mice at two different doses: 1×10^6 and 1×10^7 pfu. We included the parental wt HSV-2 as a control, but in doses 2 logs lower than that used with FusOn-H2. Toxicity was assessed by two criteria: mortality and liver function measured by monitoring levels of alanine transaminase (ALT) and aspartate transaminase (AST) activity in blood samples collected at different times after virus administration. Liver function was chosen as an endpoint because oncolytic HSV particles are distributed to the liver after systemic delivery,[21,22] and infection of both human and murine liver by HSV can cause hepatitis.[23,24] All animals receiving the wt HSV-2 died before day 10 after virus injection, in contrast to the survival of the entire group receiving FusOn-H2, even at significantly high doses (Table 2). When autopsied, the animals killed by wt HSV-2 had extensive gross necrotic foci in their livers, whereas in mice euthanized at day 4 after FusOn-H2 administration, the livers appeared normal (Figure 5a). Histologic examination of liver tissue sections confirmed these gross observations. Hepatic structures from FusOn-H2-treated mice were intact with no obvious cytopathic effects, whereas liver tissue from wt HSV-2-treated mice showed extensive necrosis and complete destruction of structural elements (Figure 5b). Immunohistochemical staining revealed widespread evidence of

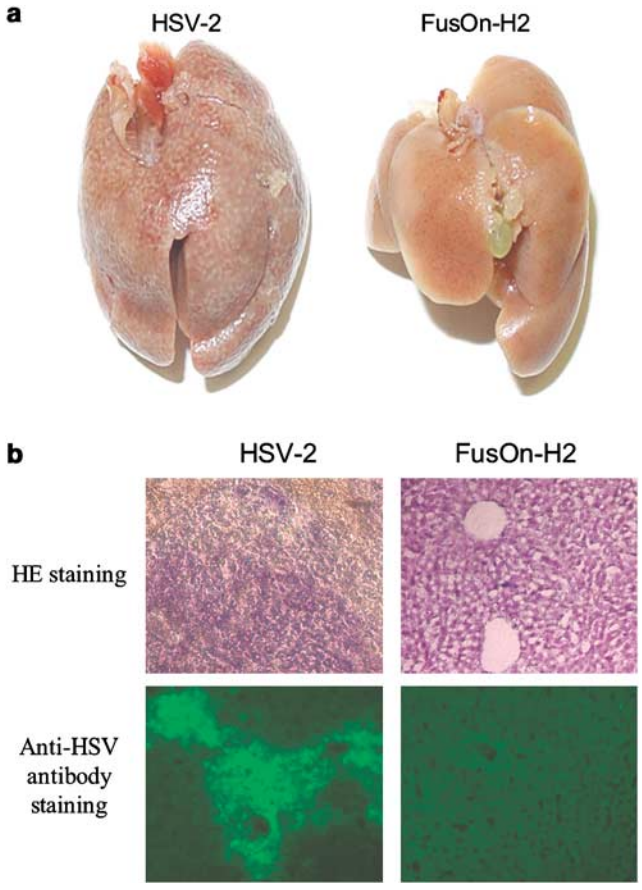

Figure 5 Evaluation of the *in vivo* toxicity of FusOn-H2. Eight-week-old immune-competent Balb/c mice were systemically injected (via the tail vein) with either FusOn-H2 or wt HSV-2 (labeled as HSV-2) at different doses ($n = 8$ mice per group). (**a**) Photographs showing the gross appearance of a typical liver from mice dying at day 3 after wt HSV-2 inoculation and mice receiving FusOn-H2 that were euthanized at day 4 after virus inoculation. (**b**) H&E staining and immunohistochemical staining (using rat anti-HSV polyclonal antibody as the first antibody and FITC-conjugated goat anti-rat IgG as the second antibody) of tissue sections prepared from the livers shown in (**a**) Original magnification $\times 200$.

Table 1 Mean number and weight of tumor nodules after virotherapy

Measure	*Treatment groups*		
	PBS	*Baco-1*	*FusOn-H2*
No. tumor nodules	6.0 ± 5.4^a	0.1 ± 0.3^b	15.5 ± 8.4
Tumor weight (g)	1.2 ± 0.6	0.7 ± 0.4^a	0.02 ± 0.05^b

Abbreviation: PBS, phosphate-buffer saline.
Tumor-free mice/total mice 0/8 1/8 7/8[b]
[a]$P < 0.01$ as compared with PBS control.
[b]$P < 0.01$ as compared with Baco-1.

Table 2 Mortality of Balb/c mice after virus injection

Virus	*Dose (pfu)*	*Injection site*	*No.surviving mice/no.injected*	*Time to death (days p.i.)*	*Observation time (week)*
wt186	1×10^4	i.v.	0/5	6,7,7,7,8	1
wt186	1×10^5	i.v.	0/5	6,6,6,7,9	1
FusOn-H2	1×10^6	i.v.	5/5		58
FusOn-H2	1×10^7	i.v.	5/5		58
wt186	1×10^4	i.c.	0/5	3,3,4,4,5	2
wt186	1×10^5	i.c.	0/5	2,2,2,3,7	2
FusOn-H2	1×10^6	i.c.	5/5		58
FusOn-H2	1×10^7	i.c.	4/5	7	58

Abbreviations: p.i., postinjection; i.v., intravenous; i.c., intracranial.

Table 3 Liver function assay after virus administration

Days	ALT (U)		ALT (U)	
	FusOn-H2	*wt186*	*FusOn-H2*	*wt186*
0	25[a]	25[a]	68[a]	68[a]
3	42	588.5[b,c]	66.5	528.5[b,c]
10	21		74	
30	34		78	

Abbreviation: ALT, alanine transaminase.
[a]The figures represent the average of ALT and AST levels obtained from five randomly bled mice before the animals were grouped.
[b]As all mice receiving wild-type virus died before the second time point, only blood samples from day 3 were available for this group.
[c]Significantly increased over pretreatment value ($p<0.01$).

viral antigen in liver treated with wt virus but only a few positive cells in liver from FusOn-H2-treated mice (Figure 5b).

In blood samples collected periodically during the first month after administration of FusOn-H2, there was only a slight increase of ALT and AST levels on day 3 post-treatment that returned to normal by the next test interval and remained there throughout the study period. By contrast, blood collected on day 3 after wt virus administration (samples were not collected at later time points as all mice died before day 10) showed a significant increase in both ALT and AST (more than 20-fold and 10-fold, respectively, $P<0.01$) by comparison with the pretreatment enzyme levels (Table 3). These data indicate that FusOn-H2 had little toxicity in the liver after systemic delivery.

Finally, we injected the viruses into the brains of immune-competent mice through stereotactic injection, again using two different doses and the parental wt virus as a control. Animal mortality was recorded up to 58 weeks after virus administration (Table 2). All animals receiving an intracranial injection of wt HSV-2 at the dose of either 1×10^4 or 1×10^5 pfu died within 1 week, whereas those receiving FusOn-H2 at 1×10^6 pfu survived for the duration of the experiment. One animal from the group injected with FusOn-H2 at a 10-fold higher dose (10^7 pfu) died on day 7 after virus administration; the rest of the animals in this group survived until the end of the experiment.

Discussion

Widespread metastasis of established primary tumors is the major factor contributing to cancer-related deaths. Patients with late-stage ovarian cancer often incur peritoneal invasion of tumor cells, usually contributing directly to their death. Hence, the peritoneal model of metastatic ovarian cancer we used in this study appears highly relevant to the clinical course of this malignant disease. Because of the relatively confined space it occupies and its ready accessibility for therapeutic intervention, the peritoneal cavity also represents an especially attractive site for investigative virotherapy. In our previous studies,[6] we used the human ovarian cancer cell line Hey8 to establish ovarian tumor xenografts in the peritoneal cavity for evaluation of potentially useful oncolytic viruses. Implantation of Hey8 cells led to the formation of fewer (1 or 2) but larger tumor nodules in the peritoneal cavity than did implantation of SKOV3 cells, which led to widespread dissemination of relatively small tumor nodules across the pelvic and abdominal surfaces, more closely resembling the clinical scenario. In the present study, two injections of FusOn-H2 at a moderate dose into the peritoneal cavity produced a clear antitumor effect, leading to complete eradication of metastases in over 87% of the animals. In sharp contrast, treatment with Baco-1, an oncolytic virus derived from HSV-1, yielded only a modest antitumor effect (one tumor-free mouse among eight tested). These results indicate that FusOn-H2 is an effective oncolytic agent for the treatment of metastatic human ovarian cancer in the peritoneal cavity.

What could account for the superior oncolytic effect of this HSV-2-derived virus? FusOn-H2, but not its parental wt virus, showed a clear fusogenic phenotype in the human breast cancer cell line MDA-MB-435.[16] Phenotypic characterization of this virus, wt186 and Baco-1, an HSV-1-based virus, on SKOV3 cells at 24 h after infection demonstrated syncytia formation by FusOn-H2 only, although by 48 h wt186 also induced weak cell-membrane fusion. As neither Baco-1 nor its wt HSV-1 parent was fusogenic in these cells[6] it appears that HSV-2 is probably inherently more fusogenic than HSV-1 in this human ovarian cancer cell line and that the mutagenesis procedure for the deletion of the PK domain enhanced the fusogenic property of the virus. Work by us and others has demonstrated that incorporation of cell membrane fusion activity into an oncolytic virus can significantly enhance its antitumor effect,[19,25] hence we attribute the potent antitumor activity of FusOn-H2 in this metastatic ovarian cancer model, at least in part, to the presence of a fusogenic property related to deletion of the PK domain from the parental HSV-2 virus.

Although the genomes of HSV-1 and HSV-2 are largely collinear and their sequences share a high homology, there are certain differences among the functions of the gene products. For example, several proteins encoded by HSV-2, including the secreted form of glycoprotein G and the virion host shutoff protein, but not their counterparts in HSV-1, are involved in evading the host's innate immunity against viral infection.[26,27] Both NK cells and interferons are key components of the host's innate immunity, which plays a crucial role in controlling HSV infection.[28–30] Thus, the HSV-2-derived FusOn-H2 mutant might have gained a therapeutic advantage over its HSV-1 counterpart (Baco-1) by virtue of mechanisms that protect it from the innate immune response of the host. This would help to explain the observation that FusOn-H2 had significantly better antitumor efficacy

than Baco-1, even though its replication capacity in tumors was about 1 log lower.

The replication capacity of FusOn-H2 depends not only on the proliferative status of the host cell, as do HSV-1-based oncolytic viruses, but also on the activation status of the Ras signaling pathway. Although *Ras* gene mutations in ovarian cancer cells are not common, the Ras signaling pathway in such cells is frequently activated through other mechanisms, such as phosphoinositide-3 kinase or protein kinase B upregulation,[31,32] making ovarian cancer an attractive target for FusOn-H2 treatment. Indeed, by exploiting both Ras signaling and cell-cycle status for its oncolytic activity, FusOn-H2 would be expected to show enhanced tumor cell-specificity and thus increased safety in patients with ovarian cancer, a prediction that is supported by the *in vivo* toxicity data collected after systemic and intracranial inoculations of FusOn-H2. Administration of the virus by either route at a relatively large dose (up to 1×10^7 pfu) was considerately less toxic than the parental wt virus given at a much lower dose, and was well-tolerated by the animals. These results indicate that FusOn-H2 is severely attenuated as compared with its parental wt186 virus and likely retains the safety profile of a conventional oncolytic HSV.

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References

1 Greenlee RT, Hill-Harmon MB, Murray T, Thun M. Cancer statistics, 2001. *CA Cancer J Clin* 2001; **51**: 15–36.
2 Landis SH, Murray T, Bolden S, Wingo PA. Cancer statistics, 1999. *CA Cancer J Clin* 1999; **49**: 8–31.
3 Martuza RL, Malick A, Markert JM, Ruffner KL, Coen DM. Experimental therapy of human glioma by means of a genetically engineered virus mutant. *Science* 1991; **252**: 854–856.
4 Mineta T, Rabkin SD, Yazaki T, Hunter WD, Martuza RL. Attenuated multi-mutated herpes simplex virus-1 for the treatment of malignant gliomas. *Nat Med* 1995; **1**: 938–943.
5 Coukos G, Makrigiannakis A, Montas S, Kaiser LR, Toyozumi T, Benjamin I *et al*. Multi-attenuated herpes simplex virus-1 mutant G207 exerts cytotoxicity against epithelial ovarian cancer but not normal mesothelium and is suitable for intraperitoneal oncolytic therapy. *Cancer Gene Ther* 2000; **7**: 275–283.
6 Nakamori M, Fu X, Meng F, Jin A, Tao L, Bast RCJ *et al*. Effective therapy of metastatic ovarian cancer with an oncolytic herpes simplex virus incorporating two membrane-fusion mechanisms. *Clin Cancer Res* 2003; **9**: 2727–2733.
7 Randazzo BP, Kesari S, Gesser RM, Alsop D, Ford JC, Brown SM *et al*. Treatment of experimental intracranial murine melanoma with a neuroattenuated herpes simplex virus 1 mutant. *Virology* 1995; **211**: 94–101.
8 Andreansky SS, He B, Gillespie GY, Soroceanu L, Markert J, Chou J *et al*. The application of genetically engineered herpes simplex viruses to the treatment of experimental brain tumors. *Proc Natl Acad Sci USA* 1996; **93**: 11313–11318.
9 Mineta T, Rabkin SD, Martuza RL. Treatment of malignant gliomas using ganciclovir-hypersensitive, ribonucleotide reductase-deficient herpes simplex viral mutant. *Cancer Res* 1994; **54**: 3963–3966.
10 Martuza RL. Act locally, think globally. *Nat Med* 1997; **3**: 1323.
11 Alemany R, Gomez-Manzano C, Balague C, Yung WK, Curiel DT, Kyritsis AP *et al*. Gene therapy for gliomas: molecular targets, adenoviral vectors, and oncolytic adenoviruses. *Exp Cell Res* 1999; **252**: 1–12.
12 Pennisi E. Will a twist of viral fate lead to a new cancer treatment? *Science* 1996; **274**: 342–343.
13 Chung TD, Wymer JP, Smith CC, Kulka M, Aurelian L. Protein kinase activity associated with the large subunit of herpes simplex virus type 2 ribonucleotide reductase (ICP10). *J Virol* 1989; **63**: 3389–3398.
14 Smith CC, Nelson J, Aurelian L, Gober M, Goswami BB. Ras-GAP binding and phosphorylation by herpes simplex virus type 2 RR1 PK (ICP10) and activation of the Ras/MEK/MAPK mitogenic pathway are required for timely onset of virus growth. *J Virol* 2000; **74**: 10417–10429.
15 Farassati F, Yang AD, Lee PW. Oncogenes in Ras signalling pathway dictate host-cell permissiveness to herpes simplex virus 1. *Nat Cell Biol* 2001; **3**: 745–750.
16 Fu X, Tao L, Cai R, Prigge J, Zhang X. A Mutant Type 2 Herpes Simplex Virus Deleted for the Protein Kinase Domain of the ICP10 Gene Is a Potent Oncolytic Virus. *Mol Ther* 2006; **13**: 882–890.
17 Fu X, Tao L, Jin A, Vile R, Brenner M, Zhang X. Expression of a fusogenic membrane glycoprotein by an oncolytic herpes simplex virus provides potent synergistic anti-tumor effect. *Mol Ther* 2003; **7**: 748–754.
18 Saeki Y, Ichikawa T, Saeki A, Chiocca EA, Tobler K, Ackermann M *et al*. Herpes simplex virus type 1 DNA amplified as bacterial artificial chromosome in Escherichia coli: rescue of replication-competent virus progeny and packaging of amplicon vectors [In Process Citation]. *Hum Gene Ther* 1998; **9**: 2787–2794.
19 Fu X, Zhang X. Potent systemic antitumor activity from an oncolytic herpes simplex virus of syncytial phenotype. *Cancer Res* 2002; **62**: 2306–2312.
20 Chen SH, Shine HD, Goodman JC, Grossman RG, Woo SL. Gene therapy for brain tumors: regression of experimental gliomas by adenovirus-mediated gene transfer *in vivo*. *Proc Natl Acad Sci USA* 1994; **91**: 3054–3057.
21 Schellingerhout D, Bogdanov Jr A, Marecos E, Spear M, Breakefield X, Weissleder R. Mapping the *in vivo* distribution of herpes simplex virions. *Hum Gene Ther* 1998; **9**: 1543–1549.
22 Wood M, Perrotte P, Onishi E, Harper ME, Dinney C, Pagliaro L *et al*. Biodistribution of an adenoviral vector carrying the luciferase reporter gene following intravesical or intravenous administration to a mouse. *Cancer Gene Ther* 1999; **6**: 367–372.
23 Ulbricht A, Farber I, Wutzler P. Herpes simplex virus hepatitis in mice: effects of treatment with trisodium phosphonoformate. *Acta Virol* 1985; **29**: 493–498.

24 Goodman ZD, Ishak KG, Sesterhenn IA. Herpes simplex hepatitis in apparently immunocompetent adults. *Am J Clin Pathol* 1986; **85**: 694–699.
25 Ebert O, Shinozaki K, Kournioti C, Park MS, Garcia-Sastre A, Woo SL. Syncytia induction enhances the oncolytic potential of vesicular stomatitis virus in virotherapy for cancer. *Cancer Res* 2004; **64**: 3265–3270.
26 Su HK, Eberle R, Courtney RJ. Processing of the herpes simplex virus type 2 glycoprotein gG-2 results in secretion of a 34,000-Mr cleavage product. *J Virol* 1987; **61**: 1735–1737.
27 Duerst RJ, Morrison LA. Herpes simplex virus 2 virion host shutoff protein interferes with type I interferon production and responsiveness. *Virology* 2004; **322**: 158–167.
28 Ashkar AA, Rosenthal KL. Interleukin-15 and natural killer and NKT cells play a critical role in innate protection against genital herpes simplex virus type 2 infection. *J Virol* 2003; **77**: 10168–10171.
29 Grubor-Bauk B, Simmons A, Mayrhofer G, Speck PG. Impaired clearance of herpes simplex virus type 1 from mice lacking CD1d or NKT cells expressing the semivariant V alpha 14-J alpha 281 TCR. *J Immunol* 2003; **170**: 1430–1434.
30 Ahmad A, Sharif-Askari E, Fawaz L, Menezes J. Innate immune response of the human host to exposure with herpes simplex virus type 1: *in vitro* control of the virus infection by enhanced natural killer activity via interleukin-15 induction. *J Virol* 2000; **74**: 7196–7203.
31 Patton SE, Martin ML, Nelsen LL, Fang X, Mills GB, Bast Jr RC *et al*. Activation of the ras-mitogen-activated protein kinase pathway and phosphorylation of ets-2 at position threonine 72 in human ovarian cancer cell lines. *Cancer Res* 1998; **58**: 2253–2259.
32 Downward J. Targeting RAS signalling pathways in cancer therapy. *Nat Rev Cancer* 2003; **3**: 11–22.